REMARKS

I. PROSECUTION HISTORY

Applicants elected claims 26, 30, and 31 (Group VII) with traverse in response to a restriction requirement mailed May 17, 2002, in which the Office alleged that the claims as filed were directed to twelve distinct inventions. In an action mailed December 3, 2002, the Examiner maintained the restriction and rejected the elected claims 26, 30 and 31 on various grounds, and additionally presented objections relating to priority and the previously submitted Information Disclosure Statement.

II. EXPLANATION OF AMENDMENTS TO THE CLAIMS

A marked-up version of the changes made to the claims can be found in Appendix A hereto. Support for the amended and new claims is found throughout the specification.

New claim 38 finds support on pages 69-78 and on page 102 of the specification. New claim 39 finds support in section "(b)" of existing claim 30, and on page 14, lines 15-18, of the specification.

New claims 40-41 find support on pages 10-14 and 57-61 of the specification. More specifically, N-terminal deletion polypeptide mutants of amino acids 1-69 of SEQ ID NO: 2, in which 1-11 residues have been deleted finds support on page 11. A polypeptide having the amino acid sequence of SEQ ID NO: 25, which includes specified amino acid substitutions at various positions, finds support on pages 11-13, and 58-60. A polypeptide having the amino acid sequence of SEQ ID NOS: 30 "MDC (n+1)," SEQ ID NO: 31 "MDC-yl," or SEQ ID NO: 32 "MDC-eyfy" finds support on pages 13 and 58. N-terminal addition polypeptide mutants find support on page 13. MDCΔPro$_2$ polypeptides find support on pages 13 and 60.

New claims 42-43 finds support in claim 26, i.e. claim 26 as originally filed. Claim 42 finds further support on page 106, lines 1-7.

The present amendment introduces no new matter. As a convenience to the Examiner, the Applicants have set forth all pending claims in Appendix B. The Applicants do not intend by these or any other amendments to abandon the subject matter of any claim as originally filed or later presented, and reserve the right to pursue such subject matter in continuing applications.

III. THE RESTRICTION OF THE CLAIMS SHOULD BE REMOVED

In paragraphs 2 and 3, the Examiner maintained the restriction requirement, stating that the Applicants' arguments were not found persuasive. In maintaining the restriction, the Examiner alleged that Vicari's murine "TECK" protein was a murine "counterpart" to human MDC of the present application, and that the presence of Vicari's murine "TECK" as prior art meant that MDC could not serve as a special technical feature common to all the claims under PCT Rule 13.2. The present application discloses a murine Macrophage-Derived Chemokine "MDC" (SEQ ID NO: 36, *e.g.*, page 97), which has a sequence unlike that of the murine chemokine disclosed by Vicari. Because Vicari does not report murine MDC, as defined and disclosed in this application, restriction on the grounds that Vicari eliminates a special technical feature is improper.

In paragraph 4, the Examiner next alleged that the various restricted groups with specific discussion of groups IV, VII, VIII, and IX, were not related to a single general inventive concept under PCT Rule 13.1. This allegation is new. In the restriction requirement, the Examiner's allegation of a lack of a general inventive concept under Rule 13.2 was predicated on there allegedly being a lack of a common special technical feature. The present amendment, which is based on Rule 13.1 independent of Rule 13.2. is not proper as Rule 13.2 serves to define the test that must be used to evaluate Unity of Invention. Rule 13.2 states that Unity of Invention as defined under Rule 13.1 is fulfilled when the claims share a common "special technical feature." Groups IV, VII, VIII, and IX, along with all the other groups, all share MDC as a special technical feature. Accordingly, the Applicants request that the entire restriction requirement be removed.

Applicants explained in their Response to the restriction requirement that the claims of groups VII-X share a common special technical feature, namely, treating conditions in a mammal using a MDC antagonist. The Examiner has not specifically addressed that point.

IV. PRIORITY TO USSN 08/939,107 HAS BEEN PROPERLY ASSERTED

In paragraph 5, the Examiner asserted that the present application repeats a substantial portion of USSN 08/939,107 filed September 26, 1997, and suggested that Applicants claim priority thereto. The Applicants have already correctly claimed priority to said application as set out in the first paragraph following the title in the specification in accordance with M.P.E.P. § 201.11 (under "Reference to First Application") and 37 C.F.R. §

1.78(a)(2)(iii). In fact, the Applicants have claimed priority back to USSN 08/479,620, filed June 7, 1995. Clarification is requested if the Examiner believes that there is a defect in the priority claim.

V. THE INFORMATION DISCLOSURE STATEMENT HAS BEEN PROPERLY FILED

In paragraph 6, the Examiner asserted that the IDS filed April 30, 2002, and entered May 6, 2002, fails to comply with 37 C.F.R. § 1.98(a)(2), because copies of the references listed in the IDS had not been provided. The Applicants filed the IDS pursuant to 37 C.F.R. § 1.98(d), which does not require the Applicants to supply copies of references that were previously provided in a parent application. However, as a courtesy to the Examiner, the Applicants are providing with this response copies of those references that the Examiner has not yet considered. Because the IDS as filed was in compliance with the rules, consideration of the references without further fees is requested. A copy of the original 1449 Form is attached. In addition, a supplementary IDS and accompanying form SB/08A have been attached herewith for consideration by the examiner.

VI. THE CLAIM REJECTIONS UNDER 35 U.S.C. § 112, SECOND PARAGRAPH, SHOULD BE WITHDRAWN

In paragraph 8, the Examiner rejected claims 26, 30 and 31 as allegedly being indefinite under 35 U.S.C. § 112, second paragraph. The Applicants respectfully traverse.

A. "Amount Effective" is Definite and Clear When Viewed in the Context of the Claims and the Specification

The Examiner alleged that claim 26 is vague and indefinite because of the phrase "amount effective." An allergic reaction is manifested by specific symptoms understood by those of skill in the art, and disappearance or amelioration of those symptoms will be recognized by such persons as evidence that the allergic reaction has been palliated. Allergy symptoms that can be palliated include common symptoms recognized by physical examination and also manifestations that can be analyzed at the cellular level by determining if the MDC antagonist has inhibited the chemotaxis of eosinophils, neutrophils, or T_H2 cells. *See, e.g.,* page 108, lines 1-5, and page 106, lines 1-7. As with most fields of medicine, the amount effective to palliate will vary depending on the subject being treated and the type and the severity of allergic reaction, but is readily determined with routine dose-response studies and/or routine monitoring of patients' symptoms. The specification (Example 33) provides an exemplary dosing of an anti-MDC antibody of 0.1 to 5 mg/kg body weight, see page 107, line

29. Accordingly, the meaning of the phrase "amount effective" is both clear and definite when viewed by one of skill in the context of the claims and the specification. The rejection should be withdrawn.

B. The Indefiniteness Rejection of Claim 30 is Moot in Light of the Amendment

Second, the Examiner alleged that the metes and bounds of "a fragment or analog of" (as recited in claim 30) is indefinite, and that if the Applicants want to claim a particular fragment or analog that they would have to indicate the exact structure of the fragment or analog. Applicants respectfully disagree with the Examiner's position. If a claim that encompasses fragments and analogs appears broad to an examiner, that is not sufficient to warrant a rejection under § 112, second paragraph. *See* M.P.E.P. § 2173.04. The various specific fragments and analogs disclosed in the present application provide sufficient support for the original "fragment or analog" language. However, in order to expedite prosecution, claim 30 has been amended so as to omit the phrase "(a) a polypeptide fragment or analog of a vertebrate MDC that inhibits MDC activation." New claims 40 and 41 define specific antagonists by structure. Thus, with the amendment of claim 30 and addition of claims 40 and 41, the basis for the rejection is moot and should be withdrawn.

Applicants reserve the right to pursue claims to other fragments and analogs in related applications.

C. Recitation of "Capable of "Is Not Vague and Carries Patentable Weight As it Defines a Specific Characteristic of the Claimed Subject Matter

Third, the Examiner alleged that the use of the phrase "capable of" in claim 30 was vague and held no patentable weight, because the phrase allegedly describes a latent characteristic of a compound or composition. The Applicants dispute the "latent" characterization, because it is understood that a compound capable of binding to MDC binds to MDC. The terms are equivalent and define a specific characteristic of the claimed subject matter. Solely for the purpose of expediting prosecution and without narrowing the claim, the Applicants amend claim 30 to read in part "a polypeptide that specifically binds a vertebrate MDC polypeptide," rendering the rejection moot.

For the reason discussed above, the rejections of claims 26, 30 and 31 under 35 U.S.C. § 112, second paragraph should be withdrawn. Any rejection of new claims 38-43 would be improper for similar reasons.

VII. THE REJECTIONS UNDER 35 U.S.C. § 112, FIRST PARAGRAPH, SHOULD BE WITHDRAWN, BECAUSE THE SPECIFICATION IS ENABLING AS FILED

In paragraph 9, the Examiner rejected claims 26, 30 and 31 under 35 U.S.C. § 112, first paragraph for alleged lack of enablement. The Applicants respectfully traverse. No undue experimentation would be necessary to practice the invention for the reasons outlined below.

A. Published Studies Demonstrate That The Specification Is Enabling

The Examiner alleged that the art at the time of Applicants' invention was "nil," and with no demonstrative, unambiguous successes in treating allergic reactions in humans with either an MDC antagonist or a TARC antagonist. While such statements support a conclusion that the present invention is both novel and non-obvious, they do not support any conclusion about enablement. *See* M.P.E.P. § 2164.02, which cites *Gould v. Quigg*, 822 F.2d 1074, 1078, 3 U.S.P.Q.2d. 1302, 1304 where the Court held "The mere fact that something has not previously been done clearly is not, in itself, a sufficient basis for rejecting an application purporting to disclose how to do it." Data published subsequent to the filing of the present application demonstrates that the invention described in the specification as filed can be practiced successfully.

Example 33, beginning on page 107 of the specification describes an antigen-induced asthma/allergy model. A mammalian subject, e.g. a mouse, is given a substance such as ovalbumin to challenge the subject's immune system and elicit an allergic reaction. A known or putative MDC antagonist compound is then administered to the subject, and the subject is monitored to see if the compound is capable of palliating the allergic reaction. One way of assessing this capability is looking for a reduction in eosinophils and/or neutrophils in the lavage fluid (fluid taken from the respiratory tract, e.g. lungs, of the subject) in experimental subjects versus control subjects.

Since the present application was filed, a number of groups have published studies that demonstrate that the invention works as described in the application.

1. Gonzalo Demonstrates the Enabling Disclosure of the Present Application.

Gonzalo, *et al.*, *Mouse Monocyte-Dervived Chemokine is Involved in Airway Hyperactivity and Lung Inflammation*, J. Immunol., 163: 403-411 (1999) (hereinafter

Gonzalo or Appendix C) is attached as Appendix C.[1] Gonzalo used an anti-MDC antibody to suppress eosinophil recruitment in a mouse model of allergy. Gonzalo generated the allergic response with ovalbumin (OVA) (*see* page 404, first column), the same allergen taught in Example 33. Gonzalo's experimental mice were pretreated with the anti-MDC antibody, consistent with Example 33 (page 107, lines 26-29). A substantial reduction of eosinophils (page 407, second column, figure 4) occurred in the experimental mice. The ability of anti-MDC antibodies to decrease the number of eosinophils, as taught in the present application (page 108, lines 1 and 2), is demonstrated by comparing the results in figure 4B for Gonzalo's control (Rb Ig) mice that received a non-MDC antibody and experimental mice that received an anti-mMDC antibody This ability of anti-MDC antibodies is further demonstrated by the specific reduction of eosinophils in the lung interstitium, an area of the lung affected by the allergen, in figure 5. (*See* figures 4 and 5 on page 409, and discussion of the same.)

Accordingly, Gonzalo demonstrates that the invention as disclosed in the present application can be practiced effectively to palliate an allergic reaction.

2. Lloyd Demonstrates the Enabling Disclosure of the Present Application.

Lloyd, *et al.*, *CC Chemokine Receptor (CCR3)/Eotaxin is Followed by CCR4/Monocyte-Derived Chemokine in Mediating Pulmonary T Helper Lymphocyte Type 2 Recruitment after Serial Antigen Challenge In Vivo*, J. Exp. Med., 191: 265-73 (2000) (hereinafter Lloyd or Appendix D) describes a study similar to that in Gonzalo with ovalbumin-challenged mice treated with anti-MDC antibodies, i.e., "neutralizing Abs." (*See* page 269, column 2.) Inspection of figure 4A of Lloyd shows that anti-MDC antibodies decreased eosinophil migration by approximately two-thirds compared to control subjects. (*See* page 272 for figure 4, and page 270 for discussion thereof.) Like the results in Gonzalo, these results also comport with those stated in Example 33.

Accordingly, Lloyd demonstrates that the invention as disclosed in the present application can be practiced effectively to palliate an allergic reaction.

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[1] "Monocyte-Derived Chemokine" as used in Gonzalo is the same chemokine as "Macrophage-Derived Chemokine" of the present application.

3. Kawasaki Demonstrates the Enabling Disclosure of the Present Application.

Kawasaki, *et al.*, *Intervention of Thymus and Activation-Regulated Chemokine Attenuates the Development of Allergic Airway Inflammation and Hyperresponseiveness in Mice*, J. Immunol., 166: 2055-2062 (2001) (hereinafter Kawasaki or Appendix E) discloses an experimental study involving a mouse allergy model, in which anti-TARC antibodies are used to cause a dramatic decrease in the number of eosinophils. Kawasaki describes the methods used for the study including the use of ovalbumin as the allergen, and application of antibody prior to induction with the allergen. (page 2056, column 1.), which mirror the methods presented in Example 33. Kawasaki states: "Treatment with anti-TARC Ab strikingly decreased the total cell number and the number of eosinophils as well as lymphocytes recovered in the lavage fluid compared with those in the group treated with control Ab (Fig. 4)." (page 2058 and in figure 4 on page 2059.) A decrease in neutrophils is also reported (*see* figure 4) in agreement with Example 33 (specification, page 108, lines 1-2).

Accordingly, Kawasaki demonstrates that the invention as disclosed in the present application can be practiced effectively to palliate an allergic reaction.

4. Bochner et al. Support a Finding that the Specification is Enabling

In the rejection, the Examiner cited Bochner, *et al.*, J. Allergy Clin. Immunol. 103:527-32 (1999) for its teachings that MDC-induced chemotaxis of eosinophils is independent of CCR3 or CCR4. This report is entirely consistent with the patent application and does not support a rejection of any claim. (*See, e.g.*, the current application's Example 12, specifically page 62, lines 16-28.) The Bochner article merely confirms that MDC acts on eosinophils, and thus supports, rather than negates, patentability by supporting the conclusion that an MDC antagonist could inhibit MDC-induced chemotaxis of eosinophils.

5. Summary

The published studies cited by the Examiner and those provided in Appendices C, D and E, alone and in combination demonstrate that the claimed invention can be practiced successfully as described in the specification. Accordingly, the rejection of claims 26, 30 and, 31 under 35 U.S.C. § 112, first paragraph should be withdrawn.

B. The Level of Skill in the Art, the Examples and the Amount of Guidance Provided Indicate that the Specification Enables One of Skill to Perform the Full Scope of the Claimed Invention

The Examiner characterized the level of skill in the art as "high." The greater the knowledge in the art about the field of the invention, the less information needs to be explicitly stated in the specification. *See* M.P.E.P. § 2164.03. The specification of the present application provides detailed guidance regarding specific inhibitors, *see, e.g.,* page 12 of the specification, and to methods of using a MDC antagonist to palliate an allergic reaction in a mammalian host, *see, e.g.,* Example 33. The detailed instructions in the specification, combined with the high level of skill in the art, means that the specification is enabling.

The Examiner further characterized the Applicants' teaching as "limited," and relied on the disclosure in Example 30, which reports that monoclonal antibodies 252Y and 252Z inhibit CCR4-mediated cellular responses to MDC, to conclude that Applicants do not teach that any or all MDC antagonists or TARC antagonists are able to inhibit any or all allergic reactions in vivo or in vitro. The Examiner also alleged that the claims are too broad in respect to a method of treating an allergic reaction by using any or all MDC antagonists or TARC antagonists. The Applicants respectfully disagree.

The presence or absence of an example is not determinative on the issue of enablement, and an applicant need not describe all actual embodiments in order to have an enabling disclosure. *See* M.P.E.P. § 2164.02. Example 33, beginning on page 107 of the specification demonstrates how MDC antagonists and TARC antagonists can be tested and used to palliate an allergic reaction. Antibodies 252Y and 252Z, described in Examples 18 may be used according to the teachings of Example 33. Moreover, the Examiner admits on page 5 of the Office Action that "[t]he monoclonal antibodies against human MDC, 252Y, and 252Z inhibit CCR4 mediated cellular response to MDC in CCR4 transfected cell lines and block the antigen-induced asthma in an animal model." That admission alone supports allowance at least for claim 31 and new claim 38.

The Examiner also alleged that, because eosinophil accumulation is CCR4 or CCR3 independent, whether the use of CCR4 is able to block the MDC-induced eosinophil accumulation is questionable and lacks supporting evidence. Whether or not MDC or TARC affects eosinophil accumulation by binding or not binding to CCR4 or CCR3 does not prevent the use of CCR4 or a fragment thereof to bind to, and consequently antagonize the normal activity of, MDC or TARC, because such activity need not be dependent on binding

to either CCR4 or CCR3. The use of CCR4 (or a fragment thereof) in such a context is as an MDC or TARC inhibitor and is analogous to the use of an anti-MDC or anti-TARC antibody. Any inhibitor that binds MDC or TARC to prevent the MDC or TARC from binding a receptor would be expected to work, including CCR4 or a fragment thereof.

Accordingly, the rejection of claims 26, 30 and 31 under 35 U.S.C. § 112, first paragraph, should be withdrawn. Similarly, any rejection of new claims 38-43 under 35 U.S.C. § 112, first paragraph, would be improper.

VIII. THE REJECTIONS UNDER 35 U.S.C. § 102(B) SHOULD BE WITHDRAWN

In paragraphs 11 and 12, the Examiner rejected claims 26 and 30 under 35 U.S.C. § 102(b) as being anticipated by Wells, *et al.* (WO 96/23068A1). The Examiner alleged that Wells, *et al.* disclose a method for using a substance of CCR4, which has 100% homology to SEQ ID NO: 34 as it is claimed in the present application to treat an allergic condition. Specific reference was made to Wells, *et al.* at page 16 lines 3-11 and claims 1-5.

While Wells, *et al.* do report using CCR4 to screen for substances that could be useful in allergy treatments, Wells, *et al.* fail to teach or suggest using CCR4 itself or fragments thereof for treating allergies. Moreover, while Wells, *et al.* disclose the use of anti-CCR4 antibodies for purification of CCR4 and for diagnostic purposes (*see* page 10, lines 27-31 of Wells, *et al.*) they do not disclose using such antibodies for treating allergic symptoms or any other diseases or conditions.

Accordingly, Wells, *et al.* do not anticipate claims 26 and 30, and the rejection should be withdrawn. Any rejection of new claims 38-43 would be improper for similar reasons.

Moreover, when one of ordinary skill considers Wells in combination with Bouchner, one would question whether the CR4-modulating agents suggested by Wells would be effective for blocking MDC-induced allergic symptoms.

SUMMARY

In view of the remarks made above, Applicants request reconsideration of the claims. Applicants submit that all pending claims, after the entrance of this amendment, are in condition for allowance and request notification of the same.

Respectfully submitted,

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APPENDIX A

Version with markings to show changes made

In the Claims:

Please amend the claims as indicated below:

26. (Amended) A method of palliating an allergic reaction in a mammalian subject, comprising the steps of:

identifying a mammalian subject in need of treatment for an allergic reaction that is characterized by eosinophil accumulation, and

administering to said mammalian subject a composition comprising an MDC antagonist compound [or TARC antagonist compound] in an amount effective to palliate the allergic reaction.

30. (Twice Amended) A method according to claim 26 wherein the MDC antagonist compound is selected from the group consisting of:

[(a) a polypeptide fragment or analog of a vertebrate MDC that inhibits MDC activation of an MDC receptor;]

(a)[(b)] an antibody that specifically binds a vertebrate MDC polypeptide;

(b)[(c)] a polypeptide [capable of binding to] that specifically binds a vertebrate MDC polypeptide and comprises an antigen-binding fragment of an anti-MDC antibody;

(c)[(d)] a polypeptide comprising the C-C chemokine receptor 4 (CCR4) amino acid sequence set forth in SEQ ID NO: 34 or comprising a continuous fragment thereof that [is capable of binding to] specifically binds MDC; and

(d)[(e)] combinations of (a)-(c)[(d)].

APPENDIX B

Pending claims upon entry of the foregoing amendment

1. (Amended) A purified polypeptide selected from the group consisting of:

(a) non-human vertebrate Macrophage Derived Chemokine (MDC) polypeptides;

(b) fragments of said non-human vertebrate MDC polypeptides that retain at least one biological activity of the MDC polypeptide; and

(c) fragments of said non-human vertebrate MDC polypeptides that are capable of inhibiting at least one biological activity of the MDC polypeptide.

2. A purified polypeptide according to claim 1 that is a non-human vertebrate MDC polypeptide or fragment thereof that retains at least one biological activity of the vertebrate MDC polypeptide.

3. A purified polypeptide according to claim 1 that is a fragment of a non-human vertebrate MDC polypeptide, said fragment being capable of inhibiting at least one biological activity of the MDC polypeptide.

4. (Amended) A purified polypeptide according to claim 1, selected from the group consisting of:

(a) a polypeptide comprising a sequence of amino acids identified by positions 1 to 68 of SEQ ID NO: 36;

(b) a polypeptide comprising a sequence of amino acids identified by positions 1 to 69 of SEQ ID NO: 38; and

(c) a polypeptide comprising a sequence of amino acids identified by positions 1 to 69 of SEQ ID NO: 46.

5. (Amended) A pharmaceutical composition comprising a purified polypeptide according to claim 1 in a pharmaceutically acceptable carrier.

6. (Amended) A purified polynucleotide comprising a nucleotide sequence that encodes a polypeptide according to claim 1.

7. A vector comprising a polynucleotide according to claim 6.

8. A host cell stably transformed or transfected with a polynucleotide according to claim 6, or with a vector comprising said polynucleotide, in a manner allowing the expression in said host cell of the polypeptide encoded by said polynucleotide.

9. A method for producing a polypeptide that is a non-human vertebrate MDC or MDC fragment or analog, said method comprising growing a host cell according to claim 8 in a nutrient medium and isolating the polypeptide from said cell or said medium.

10. An antibody that specifically binds to an MDC polypeptide, said antibody selected from the group consisting of antibody 252Y and antibody 252Z.

11. A hybridoma cell line that produces an antibody according to claim 10.

12. A kit for assaying for MDC polypeptides, said kit comprising, in association, two monoclonal antibodies that specifically bind MDC, wherein at least one of said monoclonal antibodies is a monoclonal antibody according to claim 10.

13. A method for identifying a modulator of binding between Macrophage Derived Chemokine (MDC) and an MDC receptor, comprising the steps of:

a) contacting an MDC receptor composition and a vertebrate Macrophage Derived Chemokine (MDC) polypeptide or fragment or analog thereof that binds chemokine receptor CCR4, in the presence and in the absence of a putative modulator

compound, wherein said receptor composition comprises cell membranes of cells recombinantly modified to express increased amounts of the chemokine receptor CCR4;

b) detecting binding between the receptor composition and the polypeptide; and

c) identifying a putative modulator compound in view of decreased or increased binding between the receptor composition and the polypeptide in the presence of the putative modulator, as compared to binding in the absence of the putative modulator.

14. A method for identifying a modulator of binding between Macrophage Derived Chemokine (MDC) and an MDC receptor, comprising the steps of;

a) contacting an MDC receptor composition and a vertebrate Macrophage Derived Chemokine (MDC) polypeptide in the presence and in the absence of a putative modulator compound, wherein said receptor composition comprises eosinophil cell membranes;

b) detecting binding between the receptor composition and the polypeptide; and

c) identifying a putative modulator compound in view of decreased or increased binding between the receptor composition and the polypeptide in the presence of the putative modulator, as compared to binding in the absence of the putative modulator.

26. (Amended) A method of palliating an allergic reaction in a mammalian subject, comprising the steps of:

identifying a mammalian subject in need of treatment for an allergic reaction that is characterized by eosinophil accumulation, and

administering to said mammalian subject a composition comprising an MDC antagonist compound in an amount effective to palliate the allergic reaction.

27. A method of treating a disease state characterized by aggregation of platelets in a mammalian subject, comprising the steps of;

identifying a mammalian subject in need of treatment for said disease state, and

administering to said mammalian subject a composition comprising an MDC antagonist compound or TARC antagonist in an amount effective to prevent platelet aggregation in said mammalian subject.

28. A method of treating lupus erythematosus in a mammalian subject, comprising the steps of:

identifying a mammalian subject in need of treatment for lupus erythematosus, and

administering to said mammalian subject a composition comprising an MDC antagonist compound or TARC antagonist compound in an amount effective to treat lupus erythematosus or palliate its symptoms.

29. A method of treating a disease state characterized by activation, chemotaxis, or proliferation of cells that express the chemokine receptor CCR4 in a mammalian subject, comprising the steps of:

identifying a mammalian subject in need of treatment for said disease state, and

administering to said mammalian subject a composition comprising an MDC antagonist compound or TARC antagonist compound in an amount effective to prevent at least one of activation, chemotaxis. and proliferation of cells that express the chemokine receptor CCR4 in said mammalian subject.

30. (Twice Amended) A method according to claim 26 wherein the MDC antagonist compound is selected from the group consisting of:

(a) an antibody that specifically binds a vertebrate MDC polypeptide;

(b) a polypeptide that specifically binds a vertebrate MDC polypeptide and comprises an antigen-binding fragment of an anti-MDC antibody;

(c) a polypeptide comprising the C-C chemokine receptor 4 (CCR4) amino acid sequence set forth in SEQ ID NO: 34 or comprising a continuous fragment thereof that specifically binds MDC; and

(d) combinations of (a)-(c).

31. (Amended) A method according to claim 26 wherein said MDC antagonist compound comprises an antibody substance that binds MDC, said antibody substance selected from the group consisting of monoclonal antibodies, polyclonal antibodies, single cell antibodies, chimeric, antibodies, and humanized antibodies.

32. In a vaccine compound, the improvement wherein a polypeptide is included in the vaccine composition, aid polypeptide comprising a vertebrate MDC polypeptide or biologically active fragment or analog thereof.

33. A method of stimulating an immune response in a human or animal comprising the step of administering a vaccine composition according to claim 32 to a human or animal effective to stimulate an immune response in the human or animal.

34. A method of screening a patient suspected of suffering from, or undergoing treatment for, a disorder characterized by MDC-induced T_H2 cell migration or activation, comprising the steps of:

obtaining a fluid sample from a patient suspected of suffering from a disorder characterized by MDC-induced T_H2 cell migration or activation; and

determining the concentration of MDC in the fluid sample.

35. A method according the claim 34, wherein the fluid comprises serum, and wherein the MDC concentration is determined via ELISA assay.

36. A method according the claim 34, wherein the patient is suspected of suffering from the disease state, and wherein an elevated MDC concentration is considered diagnostic of the disease state.

37. A method according to claim 34, wherein the patient is undergoing treatment for the disease state, and MDC concentration in the fluid sample is used to monitor dosing or efficacy of treatment.

38. (New) The method according to claim 26 wherein the MDC antagonist compound is a monoclonal antibody selected from the group consisting of 191D, 252Y and 252Z.

39. (New) The method according to claim 26, wherein the MDC antagonist compound is a polypeptide that specifically binds a vertebrate MDC polypeptide and comprises an antigen-binding fragment of an anti-MDC antibody.

40. (New) The method according to claim 26 wherein the MDC antagonist compound comprises a polypeptide selected from the group consisting of N-terminal deletion polypeptide mutants of amino acids 1-69 of SEQ ID NO: 2 in which 1-11 residues have been deleted, a polypeptide having the amino acid sequence of SEQ ID NO: 30 ("MDC (n+1)"), N-terminal addition polypeptide mutants of amino acids 1-69 of SEQ ID NO: 2 in which at least one amino acid residue is added, a polypeptide having the amino acid sequence of SEQ ID NO: 31 ("MDC-yl"), a polypeptide having the amino acid sequence of SEQ ID NO: 32 ("MDC-eyfy"), and MDCΔPro2 polypeptides.

41. (New) The method according to claim 26 wherein the MDC antagonist compound comprises a polypeptide having the amino acid sequence of SEQ ID NO: 25.

42. (New) A method of palliating an allergic reaction in a mammalian subject, comprising the steps of:

identifying a mammalian subject in need of treatment for an allergic reaction that is characterized by eosinophil accumulation, and

administering to said mammalian subject a composition comprising a TARC antagonist compound in an amount effective to palliate the allergic reaction.

43. (New) A method according to claim 42 wherein the TARC antagonist compound is selected from the group consisting of:

(a) an antibody that specifically binds a vertebrate TARC polypeptide;

(b) a polypeptide that specifically binds a vertebrate TARC polypeptide and comprises an antigen-binding fragment of an anti-TARC antibody;

(c) a polypeptide comprising the C-C chemokine receptor 4 (CCR4) amino acid sequence set forth in SEQ ID NO: 34 or comprising a continuous fragment thereof that specifically binds TARC; and

(d) combinations of (a)-(c).

Mouse Monocyte-Derived Chemokine Is Involved in Airway Hyperreactivity and Lung Inflammation

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The cloning, expression, and function of the murine (m) homologue of human (h) monocyte-derived chemokine (MDC) is reported here. Like hMDC, mMDC is able to elicit the chemotactic migration in vitro of activated lymphocytes and monocytes. Among activated lymphocytes, Th2 cells were induced to migrate most efficiently. mMDC mRNA and protein expression is modulated during the course of an allergic reaction in the lung. Neutralization of mMDC with specific Abs in a model of lung inflammation resulted in prevention of airway hyperreactivity and significant reduction of eosinophils in the lung interstitium but not in the airway lumen. These data suggest that mMDC is essential in the transit/retention of leukocytes in the lung tissue rather than in their extravasation from the blood vessel or during their transepithelial migration into the airways. These results also highlight the relevance of factors, such as mMDC, that regulate the migration and accumulation of leukocytes within the tissue during the development of the key physiological endpoint of asthma, airway hyperreactivity. *The Journal of Immunology,* **1999, 163: 403–411.**

Chemokines are a group of structurally and functionally related cytokines able to induce a variety of functions on hematopoietic and nonhematopoietic cells by interacting with their specific receptors (1–4). Chemokines are involved in hematopoiesis, leukocyte exocytosis, and leukocyte trafficking (5–7). This multifunctional activity results in the recruitment of leukocytes to sites of inflammation and in the secretion of membrane products and inflammatory mediators that influence tissue damage (8–11). Because chemokines are specific for particular leukocyte subsets, the selective recruitment of leukocytes to sites of inflammation is strongly directed by these molecules (4).

Over the last few years, inflammatory reactions mediating allergic lung disease have been extensively studied regarding chemokine action (12–16). In fact, several chemokines have been identified in the inflamed lung of humans, mice, and guinea pigs and have been shown to be critically active during lung allergic processes (12, 17–23). These chemokines direct the recruitment of leukocyte types such as eosinophils, lymphocytes, and monocytes that invade the asthmatic lungs (12, 14, 22, 24–26). Chemokines also deliver signals that are involved in airway hyperreactivity (AHR),[3] and it is possible to influence this physiological response by neutralizing chemokine signals at different time points during the response (27). The expression of several chemokines during lung allergic inflammatory responses is not necessarily redundant but potentially interdependent (27).

In this report, the mouse homologue of human MDC (hMDC) is described. hMDC, also described as stimulated T cell chemotactic protein-1 (STCP-1) (28), has been shown to be a potent monocyte, stimulated T lymphocyte, and activated Th2 lymphocyte chemoattractant that binds the chemokine receptor CCR4 (28–32). Here we show that murine MDC (mMDC), the expression of which is highly regulated during the course of an allergic inflammatory reaction in the lung, is produced by alveolar macrophages and smooth muscle cells, and the peptide encoded by this gene has chemoattractant activity on monocytes and stimulated lymphocytes in vitro. Neutralization of mMDC with specific Abs prevented interstitial lung inflammation and development of AHR in a murine model of lung inflammation.

Materials and Methods

Sequence database search

Sequences of known human chemokines were used to search public databases with the BLAST (basic local alignment search tool) algorithm (33). One murine clone, from the public database DBEST, containing hMDC and thymus and activation-regulated chemokine (TARC) homologous sequence (GenBank accession no. AA175762) (29, 34) was obtained from Research Genetics (Huntsville, AL) and fully sequenced. It was designated mMDC (see *Results*).

Cloning of mMDC and protein production

The full-length mMDC cDNA was cloned into mammalian expression vector pN8/ϵ (a gift from Dr. J. Morgenstern, Millennium Pharmaceuticals, Cambridge, MA) by PCR. The coding region was amplified using the following primers: 5Mend primer, 5MCGGGATCCGCCACCATGGCTAC CCTGCGTGTCCCACTCC, and 3Mend primer, 5MCGGAATTCCTAGTG GTGGTGGTGGTGGGACAGTTTATGGAGTAGCTTC.

The construct was fully sequenced to ensure the accuracy of insert. Plasmid DNA was prepared using a Qiagen (Chatsworth, CA) column and transiently transfected into 293 EBNA cells using lipofectamine (Life Technologies, Gaithersburg, MD). Forty-eight hours after transfection and 24 h before harvest of the supernatant, transfected cells were incubated with serum-free medium consisting of DMEM and OPTI-MEM in a ratio of 1:2 (Life Technologies).

Culture supernatant containing mMDC protein was applied to Ni-NTA resin in a column procedure as indicated by the supplier (Qiagen). After

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[3] Abbreviations used in this paper: AHR, airway hyperreactivity; MDC, monocyte-derived chemokine; h, human; m, murine; STCP, stimulated T cell chemotactic protein; i.n., intranasally; BAL, bronchoalveolar lavage; Penh, enhanced pause; Mch, methacholine; MCP, monocyte chemoattractant protein; TARC, thymus and activation-regulated chemokine; SDF, stromal cell-derived factor.

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washing with 25 mM imidazole, bound C-terminal-6xHis-tagged mMDC protein was eluted with 250 mM imidazole. Fractions containing recombinant mMDC protein were pooled and dialyzed against PBS.

Anti-mMDC Ab generation

Rabbit polyclonal Abs against murine mMDC were prepared according to standard methods (35). This Ab was generated against a 15-aa peptide corresponding to the N-terminal region of mMDC. The sequence of the peptide was GPYGANMEDSVCCRD. Rabbit serum was first depleted of anti-human IgG Abs by passage over a human IgG column, and anti-mMDC Abs were purified from the flow-through on an affinity column using the same peptide or using mMDC recombinant protein depending on the preparation (Research Genetics, Huntsville, AL).

Mice and in vivo procedures

C57BL/6J mice 8–10 wk old were purchased from The Jackson Laboratory (Bar Harbor, ME) and kept in the Millennium Pharmaceuticals, Inc., specific pathogen-free mouse facility. The mouse model of lung inflammation used here consists of two phases: sensitization (OVA, 0.1 mg/mouse i.p. on day 0) (Sigma, St. Louis, MO) and induction of the response (2% OVA for 5 min intranasally (i.n.) on day 8 and 1% OVA for 20 min i.n. on days 15–21) (19) (Fig. 4*A*). PBS (i.p. and/or i.n.) was administered to mice as a negative control. For the blocking experiments, mice also received 20 μg/mouse of neutralizing polyclonal Abs against mMDC. This Ab was administered i.v. 30 min before OVA provocation either on days 8 and 15 or on day 8 and days 15–21 (Fig. 4*A*). OVA-treated control mice were injected with the same amount of rabbit Ig control Ab (Rb Ig) at the same time points indicated during treatment (Dako, Carpinteria, CA). Three hours after OVA administration on day 15 or day 21, mice were sacrificed by CO_2 asphyxiation and analyzed for lung inflammation and AHR.

Bronchoalveolar lavage (BAL) was performed as described (19). AHR was expressed as enhanced pause (Penh), a calculated value, which correlates with measurements of airway resistance, impedance, and intrapleural pressure in the same mouse (36): *Penh* o (*Te/Tr* u 1) s (*Pef/Pif*) (*Te*, expiration time; *Tr*, relaxation time; *Pef*, peak expiratory flow; *Pif*, peak inspiratory flow s 0.67 o coefficient). The relaxation time is the time it takes for the box pressure to change from a maximum to a user-defined percentage of the maximum. Here, *Tr* measurement begins at the maximum box pressure and ends at 40%. AHR was measured 3 h after the last Ag challenge by recording respiratory pressure curves by whole-body plethysmography (Buxco Electronics, Sharon, CT) in response to inhaled methacholine (Mch; Aldrich Chemical, Milwaukee, WI) as described (27).

Peritoneal recruitment assays in vivo with mMDC protein were performed after injection of 800 μl i.p. of mMDC recombinant protein-containing conditioned medium or control conditioned medium. At different time points after injection (0, 1, 2, 4, and 6 h), peritoneal leukocytes were collected and enumerated. In one series of blocking experiments, mice were injected i.v. either with 20 μg/mouse of anti-mMDC neutralizing Ab or with Ab control, 30 min before mMDC recombinant protein-containing conditioned medium. Peritoneal lavage was performed 2 h after chemokine injection.

Immunohistochemical phenotyping and quantitation of leukocytes

Total BAL cell and peritoneal cell counts were performed, and aliquots (5 s 10^5 cells/slide) were pelleted onto glass slides by cytocentrifugation. To determine the number of eosinophils and neutrophils, slides were stained with Wright-Giemsa stain (Fisher Diagnostics, Pittsburgh, PA). T lymphocytes, B lymphocytes, and mononuclear phagocytes were identified by Thy 1.2 (53-2.1) (PharMingen, San Diego, CA), IgM (II/41) (Phar-Mingen), and Moma 2 (BioSource International, Camarillo, CA) staining, respectively, as described (14). Percentage of eosinophils, lymphocytes, neutrophils, and macrophages was determined by counting their number in eight high power fields (s 40 magnification; total area, 0.5 mm^2) per area randomly selected and dividing this number by the total number of cells per high power field. To obtain the absolute number of each leukocyte subtype in the lavage, these percentages were multiplied by the total number of cells recovered from the BAL fluid.

Lung sections from the different experimental groups of mice were prepared as described (14). Briefly, lungs were fixed in 10% neutral buffered formalin (J.T. Baker, Phillipsburg, NJ) and paraffin embedded. Sections (4 μm) were stained with hematoxylin and eosin according to standard protocols. An estimation of the percentage of each leukocyte subtype within the infiltrate in OVA e anti-mMDC Ab-treated mice or OVA e rabbit Ig-treated controls was made by counting 200 cells in one randomly selected peribronchiolar infiltrate and determining the number of eosinophils. Quantitation of leukocytes both in BAL fluid and in lung sections was performed in a blinded fashion.

In vitro chemotaxis

The in vitro migration of leukocytes to recombinant mMDC through an endothelial cell layer was evaluated in duplicate as described (19). The endothelial cells in transwell inserts were washed once with serum-free medium, and 2 s 10^5 leukocytes (BM cells from C57BL6/J mice, Th1 or Th2 polarized lymphocytes, eosinophils from IL-5 transgenic mice (37), or *Staphylococcus aureus* enterotoxin B (SEB)-stimulated (10 μg/ml, 12 h) or unstimulated lymph node cells from C57BL6/J mice) were added in 0.1 ml of serum-free medium. After a 2-h incubation, the Transwells were removed and the number of cells per well was counted in the FACScan by passing each sample for a constant predetermined time period (contaminating endothelial cells were gated out). In the blocking experiments, leukocytes were preincubated with either 1 or 10 μg of anti-mMDC Ab or control Ab at 37°C for 15 min before their addition to the Transwell inserts.

Generation of the HEK/mCCR4 cell line

Murine CCR4 was cloned from a mouse thymus cDNA library by PCR using primers based on the mouse CCR4 sequence (38). The full coding sequence of mCCR4 was subcloned into the mammalian cell expression vector pcDNA3.1zeo (Invitrogen). Stable cell lines were generated following transfection of the expression vector into HEK-293 cells using the calcium phosphate transfection system (Life Technologies) according to the manufacturer's instructions. Positive clones were selected with zeocin (100 μg/ml) (Invitrogen), and clones expressing high levels of mCCR4 were identified by binding to ^{125}I-labeled human TARC (Amersham).

Calcium mobilization

The ability of mMDC to activate CCR4 was determined by adding 5, 25, and 50 aliquots of the conditioned medium containing mMDC to 1 s 10^6 fura-2-loaded HEK/mCCR4 cells for each measurement, as previously described (39). Desensitization experiments were conducted by adding 50 μl of the conditioned medium containing mMDC, followed by the addition of different concentrations of human TARC or hMDC 60 s later.

Th1-Th2 cell polarization

Mice expressing the transgene for the DO11.10 αβ-TCR, which recognizes residues 323–339 of chicken OVA in association with I-A^d (40), were provided by Dr. D. Loh (Washington University, St. Louis, MO). OVA-specific TCR-transgenic $CD4^+$ T cells were isolated from the spleen (n 97% purity) by using mouse $CD4^+$ T cell subset enrichment columns (R&D Systems, Minneapolis, MN) and cultured in complete RPMI 1640 with $OVA_{323-339}$ (1 μg/ml) and mitomycin C-treated splenocytes. For Th1 phenotype development, recombinant murine IL-12 (40 ng/ml) (Endogen, Cambridge, MA) and neutralizing anti-IL-4 Ab (11B11, 20 μg/ml, R&D Systems) were added and for Th2 phenotype development recombinant murine IL-4 (50 ng/ml) and anti-IL12 (TOSH-2, 10 μg/ml, Endogen) were used. Cells were cultured for three rounds of antigenic stimulations under polarizing conditions. To determine that cells were differentiated, 2 s 10^5 cells were activated on immobilized anti-CD3 mAb (2C11, 10 μg/ml, PharMingen) in the presence of human IL-2 (10 U/ml) (Endogen) for 48 h. IL-4, IL-5, and IFN-γ levels were determined by specific ELISA (Endogen). In general, Th2 cells produced high levels of IL-4 and IL-5 but little IFN-γ, whereas Th1 cells produced high levels of IFN-γ but little IL-4 and IL-5 (Th2 cells produced 100–300 ng/ml IL-4, 50–150 ng/ml IL-5, and c 20 pg/ml IFN-γ; Th1 cells produced 7,000–15,000 ng/ml IFN-γ). The viability of Th1 and Th2 cells was n 95%. Subsequently, these cell were used for both CCR4 expression analysis and in vitro migration assays.

Measurement of mMDC protein by immunohistochemistry

mMDC protein expression was determined in both normal and inflamed mouse lung tissue with a polyclonal rabbit anti-mMDC Ab using a modified avidin/biotin staining method as described (14). Sections were overlaid with 20% normal donkey serum in PBS for 15 min and then incubated overnight at 4°C with anti-mMDC Ab diluted 1:750 in PBS with 0.1% BSA and 0.1% sodium azide. Endogenous peroxide was subsequently blocked by incubation for 20 min in methanol containing 0.3% hydrogen peroxide. Nonspecific staining due to cross-reaction with endogenous avidin or biotin was blocked by incubation with avidin solution followed by biotin solution, both for 20 min. Bound Ab was visualized by incubation

with biotinylated anti-rabbit Ig diluted in 10% normal mouse serum PBS and then with streptavidin peroxidase complex prepared according to the manufacturer's instructions (both from Dako) and incubated for 1 h each. Finally, slides were flooded with peroxidase substrate solution for 10 min before counterstaining with hematoxylin. Control slides with the following were included: 1) staining with normal rabbit Ig instead of primary Ab, 2) omission of biotinylated anti-rat Ig, and 3) omission of streptavidin complex. In addition, competitive inhibition of the Ab was accomplished by preincubation of Ab with the peptide (100-fold excess) for 45 min at 37°C before incubation with tissue sections.

Measurement of mMDC mRNA expression

mMDC mRNA expression in normal murine tissues was analyzed by Northern blot analysis. ^{32}P-labeled full-length mMDC cDNA was used to probe a murine multiple tissue Northern blot (Clontech Laboratories, Palo Alto, CA). An actin control probe was used to hybridize to the same blots to ensure that all lanes were equally loaded.

Total RNA from the lungs of OVA-treated mice or control littermates at different time points was extracted by the single-step method using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX).

mMDC mRNA expression during lung allergic inflammation was determined by Multiprobe RNase protection assay as described (27) A 464-bp mMDC probe was derived by PCR using the following primers: 5MGCTCTCGTCCTTCTTGCTGTCC-3Mand 5MAGGGGATGGAGGA GGTGAGTAAAGGTG-3MThe identity and quantity of each mRNA species in the original RNA sample was determined based on the signal intensities given by the appropriately sized, protected probe fragment bands. Values were created by expressing mMDC up-regulation relative to its expression in normal tissue. The sample loading was normalized by the housekeeping gene, GADPH, which is included in each template set.

Results and Discussion

Identification of mMDC gene

Several mouse chemokine-like sequences were identified after searching the public database DBEST. One clone contained a novel full-length CC chemokine, which was designated mMDC based on its pattern of tissue expression, chemotactic specificity in vitro, and receptor usage. Nucleotide and amino acid sequence of this clone and homology analysis are not shown because, during the preparation of our manuscript, a novel mouse chemokine named ABCD-1 showing the same gene and protein sequence was reported (41).

The mouse chromosomal location of mMDC was determined by using a panel of backcross progeny of C57BL/6J *Mus musculus* and *Mus spretus* mice. The mapping results indicated that mMDC is located on mouse chromosome 2 (data not shown).

Anti-mMDC polyclonal Ab reacting with recombinant protein

Recombinant mMDC protein was produced in 293 EBNA cells by transient transfection as described in *Materials and Methods*. Purification of 6xHis-tagged mMDC protein was made following the Ni-NTA affinity purification method (Qiagen). Affinity-purified rabbit polyclonal Abs generated against a 15-aa peptide corresponding to the N-terminal region of mMDC were shown to recognize a specific band in a Western blot against mMDC. Using this polyclonal antiserum, a single 8-kDa band was detected from the mMDC-transfected supernatant mentioned above but not in control supernatant (data not shown). This polyclonal Ab did not cross-react with murine macrophage-inflammatory protein-1α, murine monocyte chemoattractant protein (MCP)-1, mMCP-5, murine eotaxin, or stromal cell-derived factor -1α (SDF-1α) protein (data not shown). Furthermore, as shown later, in vitro leukocyte migration in response to either SDF-1α or MCP-5 was not affected by the anti-mMDC polyclonal Ab. Because it is known that in general the N-terminal regions of chemokines, such as IL-8 and MCP-1, contain receptor binding sites, it is likely that this Ab masked the receptor binding site on mMDC. This hypothesis is demonstrated below, where the neutralizing capabilities of the Ab preparation are shown.

In vitro chemotactic responses of leukocytes to mMDC

To evaluate the chemotactic function of mMDC on resting leukocytes, neutrophils, lymphocytes, and monocytes were isolated from C57BL/6J mouse bone marrow and subjected to in vitro transendothelial migration assays (42). Fig. 1*A* shows that the conditioned medium containing mMDC (but not the control conditioned medium) or purified mMDC protein induced the migration of monocytes. This migration was similar to that provoked by MCP-5 (Fig. 1*A*) and was three times smaller than that induced by SDF-1 (data not shown), which were used as positive controls. Neither bone marrow neutrophils from wild-type mice nor eosinophils from IL-5 transgenic mice (37) migrated in response to mMDC (data not shown). No effect on resting bone marrow lymphocytes in response to mMDC was detected in the same set of migration assays in which SDF-1 was the positive control (Fig. 1*A*). However, when lymph node T lymphocytes were stimulated in vitro with the superantigen *Staphylococcus aureus* enterotoxin B for 12 h (43), these cells were able to migrate in response to mMDC, showing a 3-fold increase in chemotactic index when compared with that observed in unstimulated cells (Fig. 1*B*). To further evaluate chemotactic function of mMDC on activated T lymphocyte subclasses, IL-2-stimulated Th1 and Th2 polarized cells were used in chemotaxis assays (Fig. 1*B*). Our data clearly showed that although mMDC induced both Th1 and Th2 cell migration, the chemotactic index of Th2 cells in response to mMDC was significantly higher than that observed in Th1 cells (Fig. 1*B*).

To attribute unambiguously the migration of monocytes and stimulated lymphocytes to the mMDC chemotactic activity within the serum-free supernatant from mMDC-transfected cells or to purified mMDC protein, this chemokine was blocked in vitro with affinity-purified polyclonal Abs raised against an mMDC peptide (see *Materials and Methods*). Specific anti-mMDC Ab was able to neutralize almost all of the monocyte and lymphocyte chemotactic activity of the purified mMDC protein or in the mMDC-conditioned medium (Fig. 1, *A* and *B*). Both in vitro chemotaxes induced by SDF-1α and by MCP-5 were unaffected by the anti-mMDC Ab (Fig. 1, *A* and *B*).

In vivo chemotactic responses to mMDC

To determine the efficacy of mMDC in vivo, serum-free supernatant from mMDC-transfected cells was injected into the peritoneum of mice. Maximal peritoneal leukocyte accumulation in response to mMDC was detected 2 h after injection (data not shown). At this time point, quantitation of leukocyte subtypes revealed 1-fold increase in monocyte numbers after mMDC injection (Fig. 1*C*). Peritoneal monocyte numbers (detected by Moma 2-positive cell staining) in the mMDC-treated mice and IL-8-treated control littermates were (215 y 20) s 10^3 and (140 y 12) s 10^3, respectively. This mMDC-induced monocyte accumulation in vivo correlates well with the in vitro data shown above. No differences in the total number of leukocytes or in the number of each cell type were detected when PBS-treated mice and control medium-injected mice were compared (Fig. 1*C*).

No discernible increase in peritoneal lymphocytes were detected following i.p. mMDC administration in the experimental group of mice when compared with PBS- or control medium-treated mice (Fig. 1*C*). Stimulated lymphocytes, but not resting lymphocytes, migrate in in vitro assays in response to mMDC (Fig. 1), suggesting that resting lymphocytes must be activated before responding to this chemokine and that mMDC is not able to mediate this activation.

The specificity of the migratory response to mMDC within the tissue culture supernatant injected was also confirmed in vivo by

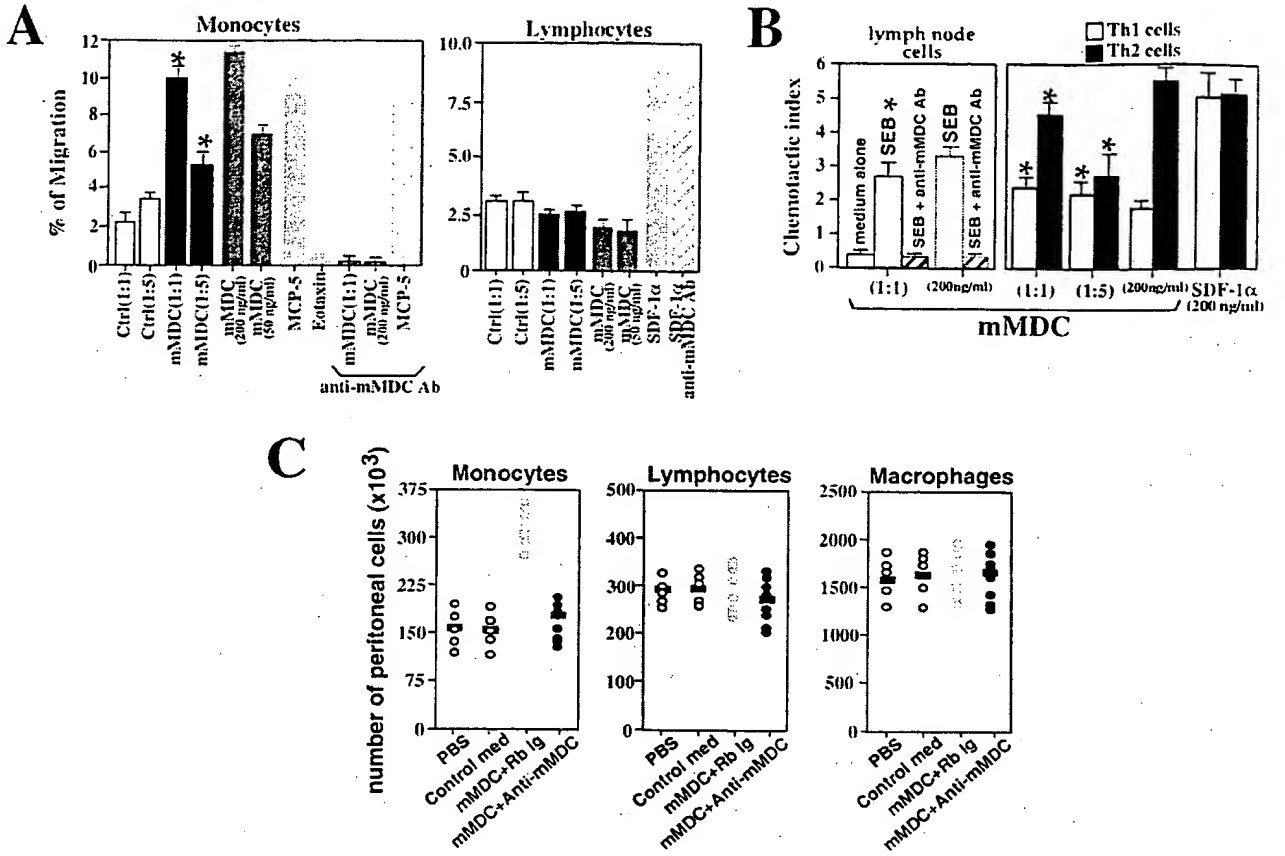

FIGURE 1. mMDC-induced leukocyte migration. *A*, Chemotactic activity of mMDC on mouse leukocytes in vitro. Bone marrow lymphocytes and monocytes were subjected to chemotaxis to different dilutions of either the conditioned medium containing mMDC (filled bars) or the control medium (open bars), or to different concentrations of purified mMDC (50 ng/ml or 200 ng/ml; gray bars). Eotaxin (200 ng/ml) was used as negative control for monocyte migration. SDF-1α (200 ng/ml) and MCP-5 (200 ng/ml) were used as positive controls for lymphocyte and monocyte migration, respectively (gray bars). These bars represent the percentage of leukocyte migration; error bars indicate the mean for three representative experiments. Hatched bars represent blockage of mMDC-induced monocyte migration by the anti-mMDC Ab as well as MCP-5 e anti-mMDC Ab and SDF-1 e anti-mMDC Ab controls. *B*, *Staphylococcus aureus* enterotoxin B-stimulated lymph node cells or in vitro polarized Th1 and Th2 lymphocytes were subjected to the same migration protocol. SDF-1α was used as a positive control for lymphocyte migration. Results are expressed as chemotactic index (the ratio between the number of cells that migrated to the sample and the number that migrated to negative control). Error bars indicate the mean for three representative experiments. Hatched bars represent blockage of mMDC-induced lymphocyte migration by anti-mMDC Ab. *, Significant difference between chemotaxis induced by conditioned medium containing mMDC or by the control medium; Student's *t* test (p c 0.01). *C*, mMDC-induced recruitment of leukocytes to the peritoneum. Peritoneal exudate was collected 2 h after injection of either mMDC-containing conditioned medium in the peritoneum of the experimental mice (filled symbols) or control conditioned medium or PBS in the peritoneum of the control littermates (open symbols). Thirty minutes before injection of mMDC-containing conditioned medium, experimental mice were injected i.v. with 20 μg/mouse of either anti-mMDC Ab (filled symbols) or Ab control (gray symbols). Each dot represents one individual mouse analyzed (5 mice per control group, 10 mice per experimental group). The bar in each panel represents the mean of the total number of cells of the leukocyte type indicated.

using anti-mMDC neutralizing Ab. No monocyte accumulation was detected after coinjection of the mMDC protein-containing supernatant and the specific neutralizing Ab against this (Fig. 1*C*).

Ca^{2+} flux in CCR4-transfectant cells and MDC cross-desensitization assays

Taken together, the previous data indicate that mMDC displays chemotactic activities on monocytes and stimulated T lymphocytes. Because of the homology between mMDC and the human chemokine MDC (29), also described as STCP-1 (28), mMDC could be considered as the mouse homologue. In fact, like mMDC, hMDC is a potent chemoattractant for monocytes and stimulated T lymphocytes (28–30, 32). In addition, hMDC has been described as a functional ligand for the chemokine receptor CCR4 (30). Corresponding to the highest expression of hCCR4 on Th2 lymphocytes vs Th1 lymphocytes, hMDC is much more active on Th2 cells than on Th1 cells (31). Similarly, mouse Th2-polarized lymphocytes, which also show higher levels of expression of CCR4 than Th1 cells by PCR (data not shown), respond more readily to mMDC in in vitro migration assays than do Th1 cells (Fig. 1*B*).

To confirm that mMDC utilizes the CCR4 receptor, calcium mobilization in response to mMDC was evaluated in HEK-293 cells transfected with mCCR4. Fig. 2 shows that calcium flux was induced in these cells following stimulation by conditioned medium containing mMDC. No calcium flux was detected when control conditioned medium was used on CCR4-transfected cells (data not shown). Mock transfectants or untransfected HEK-293 cells also did not show calcium flux following mMDC stimulation (data not shown). This indicates that, as shown for hMDC, mMDC binds CCR4 with functional consequences. Furthermore, mMDC was

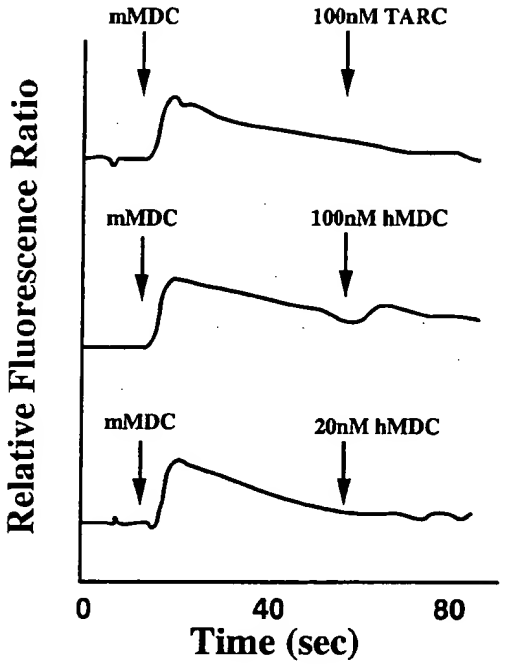

FIGURE 2. mMDC-induced calcium mobilization in cells expressing murine CCR4. HEK-293 cells stably transfected with mCCR4 were loaded with fura-2 and stimulated with 50 μl of the conditioned medium containing mMDC and either human TARC or hMDC at the indicated concentrations. Arrows indicate times of application of the chemokines.

able to desensitize CCR4, because hMDC- or TARC-induced calcium flux in CCR4-transfectant cells was abolished in a dose-dependent manner by the murine chemokine (Fig. 2).

Regulation of mMDC expression during the course of lung inflammation

mMDC mRNA expression in normal murine tissues was examined by using several multiple-tissue Northern blots probed with the mMDC cDNA. As described for hMDC (28, 29), the greatest mMDC mRNA expression was detected in the thymus and lower in the lung (data not shown and Fig. 3*A*). No or little mMDC mRNA expression was detected in the spleen, brain, liver, or kidney (data not shown and Fig. 3*A*).

Because mMDC induces the migration of monocytes, activated T lymphocytes, and Th2 cells and these leukocyte types are critical players in the evolution of inflammation, the modulation of mMDC expression in pulmonary inflammation was examined. Therefore, mMDC mRNA expression was measured by RNase protection assay in lungs isolated at different time points during OVA-induced lung allergic inflammation (19). mMDC was expressed at low levels in the lung of PBS-treated mice (Fig. 3*A*) but was up-regulated by 5-fold by day 15 of OVA treatment (Fig. 3*A*). Expression peaked at this time point but did not return to basal levels. Interestingly, day 15 correlates with the peak accumulation of monocytes/macrophages in this model and precedes the accumulation of T lymphocytes and eosinophils (see below and Fig. 4). When the expression of mMDC was analyzed at late stages (day 18, day 21) of the inflammatory response in this mouse model, it was shown that mMDC mRNA was still up-regulated but to a lesser extent than at day 15 (Fig. 3*A*). Eotaxin expression at the same time points is shown for comparison (Fig. 3*A*).

Polyclonal Ab specific for mMDC was used to determine protein expression during allergic lung disease. Low, but detectable, mMDC protein expression was observed in the lungs of PBS-treated mice, but increased mMDC expression was detected in alveolar macrophages, infiltrating macrophages, and smooth muscle cells on days 15 and 21 of OVA-treated littermates. In addition, a subset of eosinophils, mainly those confined to the alveolar spaces, stained positive for mMDC (Fig. 3*B*). The phenotype of positive cells was determined by localization and morphology.

Blockage of mMDC during lung allergic inflammation

The inflammatory response to OVA in the mouse model studied here consists of a lung accumulation (interstitium and airway lumen) of macrophages that becomes maximal at early stages of the response (monitored here 3 h after OVA challenge on day 15) and an accumulation of eosinophils and lymphocytes that reaches its plateau at late stages of the response (monitored here 3 h after OVA challenge on day 21) (14) (Fig. 4). AHR is a feature of the late stages but not of the early stages in this specific model (27).

Role of mMDC at early stages of the response. Because mMDC mRNA expression is highly up-regulated at early stages of this pathological response (day 15) (Fig. 3), mMDC neutralization experiments were performed first at these time points (days 8 and 15) during OVA treatment (Fig. 4*A*). Thereafter, mice were analyzed on day 15, coinciding with maximal infiltration of monocytes and macrophages in the lung (14). Three hours after OVA challenge on day 15, the numbers of the different leukocyte types were analyzed both in the airway lumen (BAL fluid) (Fig. 4*B*) and in the lung interstitium (lung sections) (data not shown). The specific blockage of mMDC revealed a 44% decrease in the number of BAL monocytes in response to OVA at the time point indicated (Fig. 4*B*). Blockage of macrophage/monocyte activity in this model has been shown to affect eosinophil recruitment in the lung (27). Therefore, the concomitant 50% reduction in OVA-induced BAL eosinophilia after mMDC blockage was not entirely unexpected (Fig. 4*B*). Lymphocyte or macrophage accumulation in the BAL fluid was not significantly affected by the neutralization of the chemokine (Fig. 4*B*). A reduction in eosinophil and monocyte accumulation similar to that observed in the airway lumen was found in the lung interstitium of OVA-treated mice after mMDC neutralization (data not shown). Number and phenotype of bone marrow and spleen cells were not affected during the whole treatment by the anti-mMDC Ab when compared with control littermates (OVA or OVA e Ig control Ab) (data not shown). However, because at this time point the size of the infiltrate in the interstitium is small and the content of eosinophils is low, there is no significant AHR being induced. Therefore, we could not establish the impact of mMDC blockage in AHR.

Role of mMDC at late stages of the response. To study the influence of mMDC in lung inflammation and AHR at late stages of the inflammatory response, neutralizing Abs against mMDC were administered on days 8 and 15–21 (Fig. 4*A*). Mice were analyzed 3 h after OVA challenge on day 21, coinciding with maximal infiltration of the lung by eosinophils and T lymphocytes (14). OVA-induced leukocyte accumulation in the airway lumen (as detected in the BAL) in general, and eosinophil, lymphocyte, and monocyte in particular, was not affected at late stages of the inflammatory response by mMDC neutralization (Fig. 4*C*). Similarly, the neutralization of the monocyte, lymphocyte, and eosinophil chemokine macrophage-inflammatory protein-1α during the same mouse model of inflammation does not affect monocyte and lymphocyte infiltration in the lung (27), indicating that chemokine activity is strongly regulated in vivo and may not correspond with the expected activity based on in vitro assays. This may be explained by a dominant functional role played by other chemokines, such as eotaxin, RANTES, MCP-1, and MCP-5, that are expressed at this time in the lung (27). Chemokines have been shown to

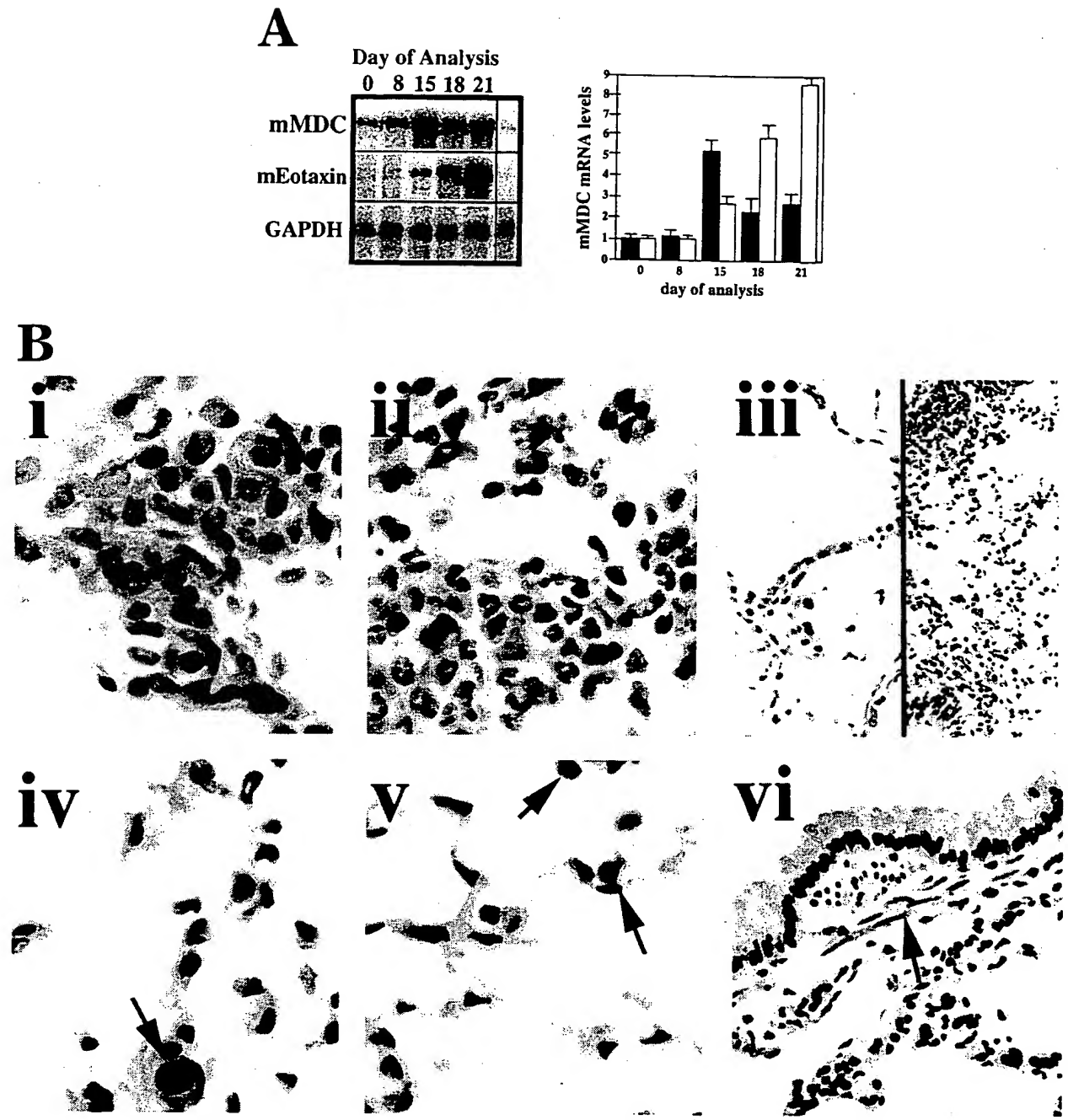

FIGURE 3. mMDC mRNA and protein expression in mouse. *A*, mMDC mRNA expression by RPA in the lung of OVA-treated mice. Inflamed lungs were obtained from mice subjected to OVA treatment 3 h after Ag challenge at the time points indicated (days 0, 8, 15, 18, and 21). mMDC mRNA expression in normal and inflamed murine lung (first five lanes) and normal kidney (final lane) is shown (*left*). Results from using a GAPDH control probe are shown in the *lower* portion of the figure. Each full bar in the *right panel* represents the mean level of mMDC mRNA expression from five mice at the time points indicated (3 h after OVA challenge) during treatment. Eotaxin mRNA expression at the same time points is also shown for comparison (*left panel*; open bars in *right panel*). Values are expressed as fold increase in mMDC or eotaxin expression, respectively, over that in PBS-treated lungs (designated a value of 1). Although mMDC is more highly expressed than eotaxin at time 0, both chemokines were normalized to 1 to better illustrate the expression pattern during disease. Values are also expressed as mean e SEM. *B*, mMDC protein expression in the lung of OVA-treated mice. Sections were prepared from lungs isolated on day 15 (*i*), 21 (*ii*), or 0 (*iii, left panel*) of OVA treatment and were stained with a polyclonal Ab that recognizes mMDC. Positive staining was detected with an avidin-biotin peroxidase staining system that resulted in a brown reaction product. Sections were counterstained with hematoxylin (blue) for contrast. Protein expression was detected in macrophages (*iv*) and smooth muscle cells (*vi*) on day 15 as well as in a proportion of infiltrating eosinophils on day 21 (*v*) (as indicated by arrows). Preincubation of the anti-mMDC Ab with the immunizing peptide (*iii, right panel*) or irrelevant control Ab did not show any staining.

coordinately activate different cellular and molecular pathways involved in the pathophysiology of asthma. Thus, MCP-1, which is not a predominantly lymphocytic or eosinophilic chemokine, diminishes both lymphocyte-derived inflammatory mediators and T cell and eosinophil recruitment to the lung of mice subjected to the same OVA model (27). Minimal OVA-induced eosinophil and T

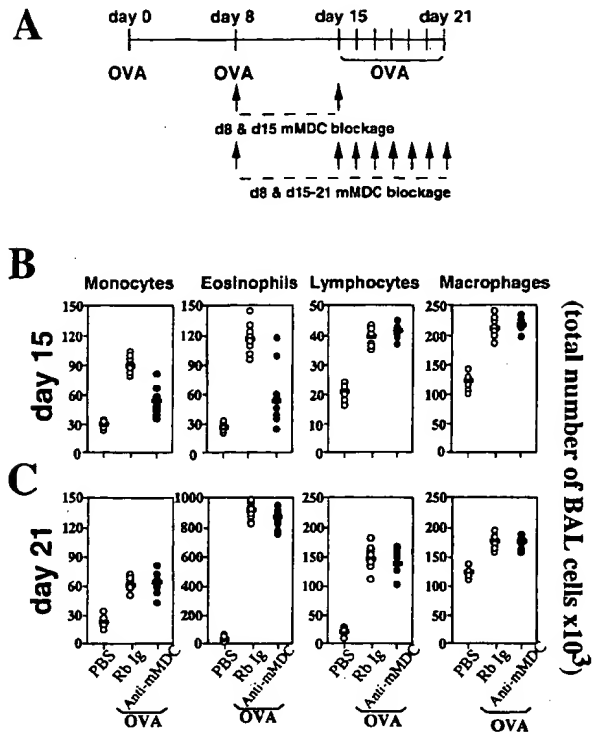

FIGURE 4. Leukocyte accumulation in the airways after mMDC blockage during lung allergic inflammation. *A*, mMDC neutralization was performed daily before each aerosolized provocation with OVA either on days 8 and 15 or on days 8 and 15–21. BAL eosinophil, monocyte, lymphocyte, and macrophage accumulation was evaluated 3 h after OVA administration on day 15 (*B*) or 21 (*C*). Each dot represents a single PBS or OVA e rabbit Ig control Ab (Rb Ig)-treated mouse (open symbol) or a single OVA e anti-mMDC Ab-treated mouse (filled symbol). Bars represent the mean of each group. One representative experiment of three, with 10 mice per group, is shown. Significant difference between control and test groups of mice was determined using the Student's *t* test (p c 0.001).

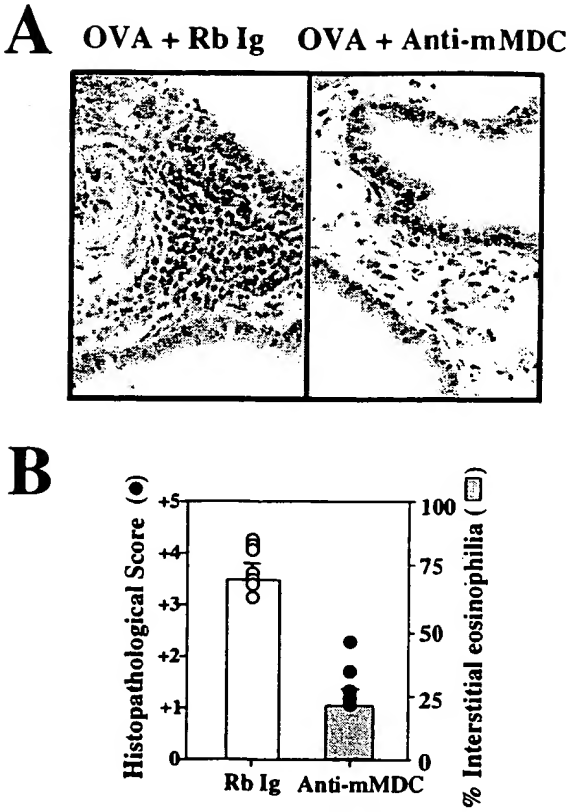

FIGURE 5. Leukocyte accumulation in the lung after mMDC blockage during lung allergic inflammation. *A*, Representative sections of lungs isolated from mice treated with OVA e rabbit Ig control Ab (Rb Ig) or OVA e anti-mMDC Ab are shown. *B*, Semiquantitative scoring system was used to estimate the size of lung infiltrates, where e 5 signifies a large widespread infiltrate around the majority of vessels and bronchioles, and e 1 signifies a small number of inflammatory foci. Each dot represents a single OVA e anti-mMDC Ab-treated mouse (●) or OVA e rabbit Ig-treated control (○). An estimation of the percentage of eosinophils within the infiltrate in OVA e anti-mMDC Ab-treated mice (filled bar) or OVA e rabbit Ig-treated controls (open bar) was made by counting 200 cells in one randomly selected peribronchiolar infiltrate and determining the number of eosinophils present (*B*). Values are expressed as the mean e SEM.

lymphocyte accumulation is detected in the lung interstitium after the neutralization of the monocytic chemokine MCP-5, whereas BAL eosinophil and T lymphocyte numbers are not affected under the same conditions (27). Interestingly, the examination of tissue sections of OVA-treated mice after blockage of mMDC revealed a differential role for the chemokine mMDC in eosinophil accumulation in the lung interstitium. During this phase of the response, there is a substantial interstitial infiltrate that develops in perivascular and peribronchiolar areas, which was inhibited by 70% after the administration of anti-mMDC Ab (Fig. 5). This reduction in infiltrate size was seen to be due to a significant decrease in numbers of eosinophils within the infiltrate (Fig. 5). OVA-induced lymphocyte accumulation is not markedly affected by mMDC neutralization. This finding deserves further comment; despite the significant chemotaxis in vitro of both Th2 and activated lymphocytes to mMDC, no obvious effect on lymphocyte recruitment is observed in vivo during OVA challenge following mMDC neutralization. This could be due to the number of Ag-specific Th2 cells that migrate to the lung in this active immunization model. These cells represent a small fraction of the total lymphocyte accumulation, which makes it difficult to detect a reduction in the total cell number. In fact, we are currently studying a lung inflammation model based on the adoptive transfer of TCR-transgenic Th2 cells into mice. In this model, Ag-specific Th2 cells can be monitored following their arrival to the lung interstitium and airway lumen during the response. Our results show that mMDC is responsible for 80% of the Th2 cells that migrate to this organ. However, Ag-specific Th2 cells represent c 15% of all the T cells present in the lung in this adoptive transfer model (C. M. Lloyd et al., manuscript in preparation). These findings suggest that the percentage of Th2 cells in the active immunization model used here could be significantly lower and therefore difficult to detect. Alternatively, this could also be explained by the fact that mMDC might influence a small subset of Ag-specific cells by promoting their retention in a specific interstitial location, a phenotype that will be difficult to substantiate in this model. These findings suggest that mMDC is instrumental in the accumulation of eosinophils within the lung interstitium during allergic eosinophilia by altering the trafficking/retention of the eosinophils and monocytes through the lung interstitium rather than affecting their extravasation from the blood vessels or their exit to airway lumen.

Role of mMDC in the induction of AHR

To determine whether the reduction of inflammation in the lung interstitium, but not in the airway lumen, following mMDC neutralization is associated with changes in airway function, AHR was evaluated in OVA-treated mice after mMDC blockage. Fig. 6

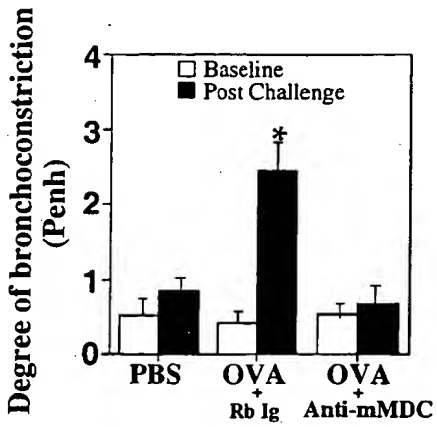

FIGURE 6. Inhibition of OVA-induced AHR after mMDC blockage. Results are shown as the mean y SEM for Penh before (open bars) and after (filled bars) Mch provocation (n o 10, three independent experiments). Mice were exposed to an aerosol of Mch for 1 min, and airway constriction was evaluated for the next 5 min. Mice treated with PBS or OVA e rabbit Ig were used as controls for OVA-treated littermates in which mMDC was blocked from days 8 to 21. Significant difference between control and test groups of mice was determined using the Student's t test (p c 0.01) and is indicated by an asterisk.

shows that mMDC neutralization inhibits the development of AHR in this experimental group of mice when compared with OVA control-treated mice. This decrease correlates with the 70% reduction in leukocyte accumulation, mainly eosinophils, in the lung interstitium of OVA-treated mice after mMDC blockage. This suggests a correlation between the location of the inflammatory cells in the lung and the establishment of AHR. Evidence indicates that eosinophils accumulated in the airways of asthmatic patients dictate the severity of disease (44). However, it has also been reported that the development of AHR depends on the recruitment of eosinophils to the mouse bronchial submucosa, but not to the airways (45). In addition, the blockage of the chemokine MCP-5, which affects interstitial eosinophil recruitment but not airway eosinophilia, abrogates AHR (27). Likewise, a recent clinical study has found no significant correlation between the degree of AHR and the number of inflammatory cells in sputum or BAL (46).

Concluding remarks

The murine model studied here has allowed us to dissect the predominant features of allergic lung disease, namely, cellular inflammation and bronchial hyperreactivity. Disease development necessitates the migration of leukocytes from the peripheral circulation through the vascular endothelium, across the lung interstitium, and into the airway lumen via the bronchial epithelium. We have previously determined that chemokines act in a tightly controlled, coordinated fashion to direct migration of leukocytes through the interstitium (as observed in histology) and into the airway lumen (as observed in lavage). The data presented here indicate that the presence of eosinophils (and, indeed, other inflammatory cells) in the airway lumen is not sufficient to induce the AHR that is characteristic of asthmatic processes. Rather, our data indicate that eosinophils and other inflammatory cells have to be localized within the peribronchiolar submucosa or in perivascular regions (as detected in sections of the lung interstitium) to induce these deleterious effects.

Our data support the notion that the chemokine mMDC is critical for the retention/trafficking of eosinophils and other leukocytes and for their proper localization to these areas in the lung during the development of an allergic reaction. During preparation of our manuscript, a mouse novel chemokine named ABCD-1 showing the same nucleotide and amino acid sequence was reported (41). This recent study describes ABCD-1 as the first activated T cell chemokine produced in large amounts by activated B cells. This suggests the possible implication of mMDC/STCP-1/ABCD-1 during a T cell-dependent B cell-humoral response.

The finding that mMDC is able to induce the recruitment of effector or regulator leukocytes during the allergic reaction makes the findings mentioned above even more relevant for the development of disease.

Finally, although there is no synteny between the chromosomal location for mMDC and hMDC loci (mouse chromosome 2 (data not shown) and human chromosome 16 (29) respectively), based on sequence homologies, pattern of tissue expression, receptor usage, and functional activities, mMDC may be considered the murine homologue of hMDC/STCP-1.

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CC Chemokine Receptor (CCR)3/Eotaxin Is Followed by CCR4/Monocyte-derived Chemokine in Mediating Pulmonary T Helper Lymphocyte Type 2 Recruitment after Serial Antigen Challenge In Vivo

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Abstract

Isolated peripheral blood CD4 cells from allergic individuals express CC chemokine receptor (CCR)3 and CCR4 after expansion in vitro. In addition, human T helper type 2 (Th2) cells polarized in vitro selectively express CCR3 and CCR4 at certain stages of activation/differentiation and respond preferentially to the ligands eotaxin and monocyte-derived chemokine (MDC). However, controversy arises when the in vivo significance of this distinct expression is discussed. To address the functional role of CCR3/eotaxin and CCR4/MDC during the in vivo recruitment of Th2 cells, we have transferred effector Th cells into naive mice to induce allergic airway disease. Tracking of these cells after repeated antigen challenge has established that both CCR3/eotaxin and CCR4/MDC axes contribute to the recruitment of Th2 cells to the lung, demonstrating the in vivo relevance of the expression of these receptors on Th2 cells. We have shown that involvement of the CCR3/eotaxin pathway is confined to early stages of the response in vivo, whereas repeated antigen stimulation results in the predominant use of the CCR4/MDC pathway. We propose that effector Th2 cells respond to both CCR3/eotaxin and CCR4/MDC pathways initially, but that a progressive increase in CCR4-positive cells results in the predominance of the CCR4/MDC axis in the long-term recruitment of Th2 cells in vivo.

Key words: chemokines • effector T helper type 2 cells • migration • allergic airway disease • chemokine receptors

Introduction

T cells are critical mediators of inflammation, and as such their migration to inflammatory sites is a tightly controlled process involving a complex series of molecules expressed by a variety of cell types. This results in the delivery of functional subsets of cells to particular tissues or microenvironments. This is especially important for T cells, since effector T cells can be divided into distinct subsets based on their cytokine profiles and functional properties. Th1 cells characteristically produce IFN-γ and contribute to host defense against pathogens, whereas Th2 cells produce IL-4 and IL-5 and are associated with allergic reactions involving IgE, eosinophils, and basophils (1). Th2 cells and the cytokines they secrete are thought to be critically important for the development of injury during allergic reactions such as asthma. However, it is unclear how or why the Th2 subset migrates to the lung. Th2 cells have previously been distinguished from Th1 cells by virtue of their cytokine profile, although more recently a range of surface markers specific for Th2 cells has been defined (2, 3). Of particular importance to the question of selective Th subset migration to inflammation sites is the growing evidence that chemokine receptor expression is tightly regulated on Th cells, and that Th cell subsets express restricted receptors for chemokines (4). In accordance with this selective expression, Th1 and Th2 cells differentially migrate in response to the chemokines that bind to these receptors.

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Eotaxin and monocyte-derived chemokine (MDC)[1] are among the chemokines that seem to attract selectively Th2 but not Th1 cells (5, 6). Eotaxin is produced by epithelial cells and binds CC chemokine receptor (CCR)3 with high affinity and fidelity (7–9), whereas MDC is produced by macrophages and interacts specifically with CCR4 (10). CCR3 was originally described on eosinophils and basophils (8, 9), but has been documented as being present on human Th2 but not Th1 cells (5, 6). The fact that this particular Th cell subset expresses the receptor for a proeosinophilic chemokine is interesting in the context of the pathophysiology of allergic lung disease. The attraction of these Th2 cells by eotaxin may represent a mechanism by which an allergen-driven reaction escalates with the production of IL-4 and IL-5, both of which are necessary for the differentiation and activation of eosinophils. CCR4 has been similarly identified as a Th2-specific marker (11, 6), and its ligands, MDC and thymus and activation-regulated chemokine (TARC), have been shown to attract Th2 cells in preference to Th1 cells (6, 11–13). CCR8 is also selectively expressed on Th2 cells (14). Several groups have used T cells, either directly isolated from patients or generated in vitro, to show elegantly that CCR3 and CCR4 identify a subset of human T cells that exhibit a cytokine profile consistent with that of Th2 cells. However, the role of the CCR3/eotaxin or CCR4/MDC axes in attracting effector Th2 cells has not been established during in vivo inflammatory processes.

The aim of this study was to determine the functional importance of the eotaxin/CCR3 and MDC/CCR4 axes on the migration of antigen-specific Th2 cells in vivo using a T cell transfer model of allergen-induced lung injury in mice. We have used this in vivo model to show that eotaxin/CCR3 and MDC/CCR4 interactions play a critical, cooperative role in the homing of antigen-specific Th2 cells to the challenged lung, giving rise to eosinophilia and bronchial hyperresponsiveness (BHR). Moreover, we have determined that eotaxin/CCR3-mediated recruitment of Th2 cells in vivo is transient and progresses to an MDC/CCR4-dominated response that is maintained over time.

Materials and Methods

Mice. Mice expressing the transgene for the DO11.10 TCR-α/β, which recognizes residues 323–339 of chicken OVA in association with I-Ad, were provided by Dr. D. Loh (Washington University, St. Louis, MO [15]). Transfer recipients were 6–8-wk-old female BALB/cJ mice (The Jackson Laboratory).

In Vitro Polarization of T Cells. OVA-specific TCR-transgenic $CD4^+$ T cells were isolated from the spleen and cultured in complete RPMI 1640 medium with OVA323–339 (1 μg/ml) and mitomycin C–treated splenocytes. For Th1 phenotype development, recombinant murine IL-12 (40 ng/ml; Endogen) and neutralizing anti–IL-4 Ab (11B11, 20 μg/ml; R&D Systems) were added; for Th2 phenotype development, recombinant murine IL-4 (50 ng/ml) and anti–IL-12 (TOSH-2, 10 μg/ml; Endogen) were used. Cells were cultured for three rounds of antigenic stimulation under polarizing conditions. At this point, the cells were divided into two portions, with the majority being used to induce pulmonary inflammation as described below. A small sample (2×10^5 cells) from each culture was activated on immobilized anti-CD3 mAb (2C11, 10 μg/ml; PharMingen) in the presence of human (h)IL-2 (10 U/ml; Endogen) for 48 h to determine the integrity of the polarization. Culture supernatants were collected for measurement of IL-4, IL-5, and IFN-γ levels by ELISA (Endogen), and cell pellets were collected for RNA extraction and PCR analysis. Th2 cells produced high IL-4 and IL-5 levels but little IFN-γ, whereas Th1 cells produced high IFN-γ levels but little IL-4 and IL-5 (Th2 cells: 100–300 ng/ml IL-4, 50–150 ng/ml IL-5, and <20 pg/ml IFN-γ; Th1 cells: 7,000–15,000 ng/ml IFN-γ). Similarly, RNA expression analysis revealed that Th2 cells expressed predominantly IL-4 and IL-5 but little if any IFN-γ, whereas the reverse was true of Th1 cells.

Induction of Pulmonary Inflammation. In preparation for induction of allergic inflammation, Th1 or Th2 cells produced as described above were rested in hIL-2 (10 U/ml; Endogen) for 48 h before being washed in tissue culture medium. Recipient BALB/c mice were given 2×10^6 cells intravenously. 24 h later, mice were exposed to an aerosol of OVA (50 mg/ml, Grade V; Sigma Chemical Co.) for 20 min. Thereafter, mice were challenged daily and were killed 24 h after the last aeroallergen challenge on day 4, 7, or 14. Control mice received cells but were challenged with aerosolized PBS. After the mice were killed, bronchoalveolar lavage (BAL) was collected by cannulation of the trachea and lavage with 1 ml of PBS. Lungs were then inflated with optimum cutting temperature (OCT) compound and removed, and the right lobes of the lung were snap-frozen in liquid nitrogen while the left were fixed in 10% buffered formalin.

For blocking studies, mice were injected daily with 100 μg per mouse polyclonal rabbit antieotaxin Abs (7) or polyclonal rabbit anti–murine MDC (13) 30 min before OVA challenge. Mice were then killed at day 4 after T cell transfer (after three antigen challenges) or at day 7 (after six antigen challenges), as shown in Fig. 1.

Determination of BHR. Airway responsiveness was measured in Th2 recipient mice 24 h after the last aerosol challenge by recording respiratory pressure curves using whole body plethysmography (Buxco; EMKA Technologies) in response to inhaled methacholine (Sigma Chemical Co.) at concentrations ranging from 2.5 to 25 mg/ml for 1 min. Airway responsiveness was expressed in enhanced pause (P_{enh}), a calculated value that correlates with measurement of airway resistance, impedance, and intrapleural pressure in the same mouse: $P_{enh} = (t_e/t_r) \times Pef/Pif$ (t_e, expiration time; t_r, relaxation time; Pef, peak expiratory flow; Pif, peak inspiratory flow).

BAL. Total BAL cells were counted, and aliquots (5×10^5 cells per slide) were pelleted onto glass slides by cytocentrifugation. A differential cell count was then performed after Wright-Giemsa staining (Fisher Diagnostics). Percentages of eosinophils, lymphocytes, neutrophils, and macrophages were determined by counting in eight randomly selected high-power fields (hpf; magnification: ×40; total area: 0.5 mm^2) and dividing this number by the total number of cells per hpf. To obtain the absolute number of each leukocyte subtype in the lavage, these percentages were multiplied by the total number of cells recovered from the BAL fluid.

In Vivo Measurement of Cytokine Production. Levels of the cytokines IL-4, IL-5, IFN-γ, IL-6, and IL-10 were determined in the lavage fluid of mice using ELISA kits (Endogen).

[1] *Abbreviations used in this paper:* AAD, allergic airway disease; BAL, bronchoalveolar lavage; BHR, bronchial hyperresponsiveness; CCR, CC chemokine receptor; hpf, high-power field(s); MDC, monocyte-derived chemokine; P_{enh}, enhanced pause; TARC, thymus and activation-regulated chemokine.

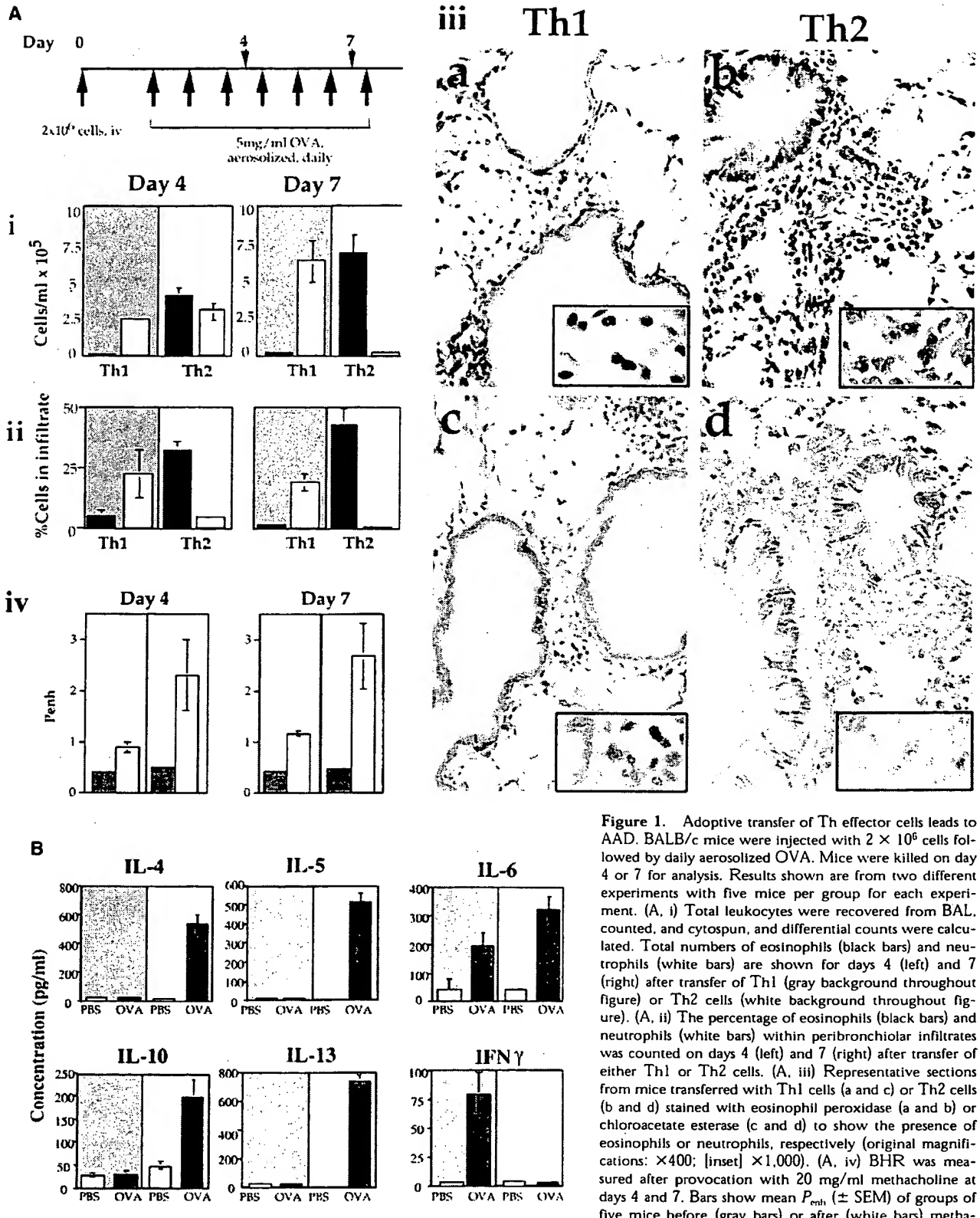

Figure 1. Adoptive transfer of Th effector cells leads to AAD. BALB/c mice were injected with 2×10^6 cells followed by daily aerosolized OVA. Mice were killed on day 4 or 7 for analysis. Results shown are from two different experiments with five mice per group for each experiment. (A, i) Total leukocytes were recovered from BAL, counted, and cytospun, and differential counts were calculated. Total numbers of eosinophils (black bars) and neutrophils (white bars) are shown for days 4 (left) and 7 (right) after transfer of Th1 (gray background throughout figure) or Th2 cells (white background throughout figure). (A, ii) The percentage of eosinophils (black bars) and neutrophils (white bars) within peribronchiolar infiltrates was counted on days 4 (left) and 7 (right) after transfer of either Th1 or Th2 cells. (A, iii) Representative sections from mice transferred with Th1 cells (a and c) or Th2 cells (b and d) stained with eosinophil peroxidase (a and b) or chloroacetate esterase (c and d) to show the presence of eosinophils or neutrophils, respectively (original magnifications: ×400; [inset] ×1,000). (A, iv) BHR was measured after provocation with 20 mg/ml methacholine at days 4 and 7. Bars show mean P_{enh} (± SEM) of groups of five mice before (gray bars) or after (white bars) methacholine stimulation. (B) BAL cytokines were measured by ELISA in OVA- (gray bars) and PBS-challenged mice (white bars) after transfer of Th1 (gray panel) or Th2 cells (white panel). Each bar represents the mean (± SEM) concentration in groups of five mice on day 4.

Measurement of Chemokine Ligand and Receptor Expression by PCR Analysis. PCR was performed using the Advantage® KlenTaq polymerase (Clontech Laboratories) according to the manufacturer's instructions. cDNA derived from 25 ng of total RNA was used for each 30-μl reaction containing 0.5 μM primers, 0.2 mM dNTP mix, 1× PCR reaction buffer, and 0.5 μl polymerase. Samples were amplified at 94°C for 30 s, 52–60°C for 1 min, and 68°C for 1 min for 20, 25, or 32 cycles. 10 μl of each reaction was loaded per well on 1.5% agarose gels. Primer sequences were as follows: for CCR3, 5′-TCTGTGGAATGAGTGGGGTTTTG and 5′-GTAATACGACTCACTATAGGGACTTCTGGATAGCGAGGACTG; for CCR4, 5′-ATCGTGCACGCGGTATTCTCC and 5′-GACGGGGTTAAGGCAGCAGTGA; for MDC, 5′-GGTGAAGAAGCTACTCCATAAACT and 5′-GTAATACGACTCACTATAGGGAGAAGGGATAGAGGGGAGGTA; and for eotaxin, 5′-TCTCCCTCCACCATGCAGAG and 5′-CAGATCTCTTTGCCCAACCT. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were purchased from Clontech Laboratories.

Lung Histology and Immunohistochemistry. The left lobe of the lungs was fixed in 10% neutral buffered formalin (NBF; J.T. Baker) and paraffin embedded. Sections (4 μm) were stained for cyanide-resistant peroxidase according to standard protocols (16), then counterstained with hematoxylin to depict eosinophils or with chloroacetate esterase to show neutrophils. The composition of infiltrates was then determined by counting the total number of infiltrating cells in five peribronchiolar fields and determining the percentage of eosinophils, neutrophils, and mononuclear cells. General morphology was assessed on hematoxylin and eosin–stained sections.

For determination of antigen-specific T cells within lung tissue, serial frozen sections (4 μm) were stained with either anti-CD4 (PharMingen) or an Ab specific for the transgenic TCR, KJ126 (17). Both of these Abs were biotinylated, and positive staining was detected using streptavidin-peroxidase (DAKO Corp.) followed by diaminobenzidine (DAB; Vector) before counterstaining in hematoxylin. Eosinophils were stained for cyanide-resistant peroxidase as described above.

The number of cells per hpf was obtained by counting positively stained cells (CD4 cells or eosinophils) in five fields per section at a magnification of 400. To calculate the percentage of KJ126$^+$ CD4 cells, CD4 cells were counted; the same field was located on the KJ126-stained serial section, and positive cells were enumerated. At least 250 CD4 cells were counted for each section, and the percentage of KJ126$^+$ CD4 cells was then calculated.

CCR3$^+$ or CCR4$^+$ Th2 cells were detected in lung sections by immunohistochemical staining using polyclonal Abs specific for the COOH terminus of CCR3 or CCR4 (Santa Cruz Biotechnology). Staining of primary Abs was visualized with a biotinylated donkey anti-goat Ig Ab (Jackson Immunochemicals) followed by streptavidin-peroxidase as described above. Positively stained Th2 cells were enumerated by locating an infiltrate in the serial KJ126-stained section and counting the percentage of KJ-stained cells that were either CCR3$^+$ or CCR4$^+$. At least 50 KJ126-stained cells were counted in each section from lungs obtained at days 4 ($n = 6$), 7 ($n = 4$), and 14 ($n = 4$), with between two and three lobes stained per mouse.

Results and Discussion

Adoptive Transfer of Th Effector Cells Leads to Allergic Airway Disease. The evaluation of the role of chemokines in the migration of Th2 cells in vivo during active immunization models of allergic airway disease (AAD) is complicated by the difficulty of tracking a small subset of effector Th2 cells that are specific for antigen during the course of the inflammatory response. To evaluate the role of the eotaxin and MDC chemotactic pathways in vivo, we sought a mouse model in which basic pathophysiological features of AAD (eosinophilia, interstitial inflammation, bronchial hyperresponsiveness, and cytokine production) could be induced in vivo upon transfer of well-characterized, easy-to-track, antigen-specific Th2 cells. Such an approach would enable us technically to address the hypothesis mentioned above, but interpretation of the results obtained would necessarily have to consider the clear differences and limitations of the experimental system chosen. Specifically, a mouse model system in which some of the important processes during the development of an AAD-type chronic inflammatory reaction are totally or partially "bypassed," whereas others (i.e., the migration, accumulation, and activation of Th2 cells to the airways and the pathophysiologies they elicit) are presumably maintained. Therefore, it is important to recognize when interpreting the data presented in this report that antigen presentation, antigen-presenting cell activation and migration, and activation, migration, and differentiation of naive T cells and that of their immature effector Th descendants, among others, are essential processes that most likely occur and progress differently in adoptive transfer models and active immunization models of AAD.

Several elegant models of AAD have been described whereby transfer of in vitro–polarized Th2 cells induces pulmonary eosinophilia (18–21). However, the Th cells used for these studies were, in general, polarized for short times in culture. Therefore, we set out to develop a system whereby Th cells were maximally polarized to ensure differential chemokine receptor expression, and thus induced multiple pathophysiological endpoints after transfer in vivo. Th2 or Th1 cells were generated in vitro after several rounds of polarizing cytokines before transfer in vivo, when mice received multiple serial in vivo antigen challenges. Accordingly, Th1 or Th2 cells were transferred intravenously to unsensitized BALB/c recipient mice, and changes in lung function were measured at various time intervals after antigen challenge. Mice were then killed at day 4 or 7, and the extent of inflammation was determined in the BAL and tissue (Fig. 1). Control mice that received cells but no antigen challenge showed no increase in cells either in the BAL or the tissue. Transfer of either Th1 or Th2 cells in conjunction with serial OVA challenge resulted in an increase in the total number of lavage leukocytes, as has been reported previously using similar protocols (18, 21). Staining of cytospins revealed a differential migration of leukocytes to BAL after Th2 transfer compared with Th1 transfer, in that transfer of Th2 cells initiated an eosinophilic infiltrate, whereas Th1 transfer initiated a neutrophilic infiltrate. Infiltration increased with challenge, peaking at day 7 (Fig. 1 A, i). Similar results were observed in lung tissue, in which Th1 transfer resulted in a perivascular and peribronchiolar infiltrate, composed largely of neutrophils, in conjunction with macrophages and lymphocytes. Conversely, Th2 transfer initiated

an eosinophilic infiltration to perivascular and peribronchiolar areas (Fig. 1 A, ii). The proportion of either eosinophils or neutrophils was 35% after Th2 cell transfer and 25% after Th1 cell transfer, respectively (Fig. 1 A, iii). Interestingly, this induction of pulmonary inflammation after transfer of antigen-specific Th2 cells was accompanied by a corresponding increase in BHR (Fig. 1 A, iv), although no such change was detected after Th1 cell administration. The polarized pathological response to transfer of Th1 or Th2 cells was reflected in the repertoire of cytokines in BAL fluid. Th1 cell transfer induced secretion of IFN-γ but low levels of IL-4, -5, -10, and -13, whereas Th2 transfer was associated with an increase in IL-4, -5, -6, -10, and -13, with no discernible increase in IFN-γ (Fig. 1 B, i).

Th2 Cells Preferentially Express CCR3 and CCR4 In Vivo As Well As In Vitro. Recent evidence has shown that effector Th cells are polarized with respect to their chemokine receptor expression as well as their cytokine production, and that Th2 cells preferentially express CCR3 and CCR4 (5, 6, 11). These findings were first confirmed in the murine in vitro–polarized Th cells used for this study. Th2 cells showed increased expression of CCR3 and CCR4 RNA after activation with anti-CD3 and IL-2 (Fig. 2 A). The increased expression of CCR3 after activation correlates well with an enhanced calcium-mobilization response to eotaxin stimulation on mouse activated Th2 cells (not shown) compared with nonactivated control cells. This is somewhat unexpected, as anti-CD3 stimulation, in contrast to CCR4, downregulates CCR3 expression in human Th2 cells (14). This difference could reflect a disparity between the mouse and the human system, or simply represent a different degree of differentiation/activation between the cells used in the experiments described here and those used by other investigators. Immunohistochemical staining for CCR3 and CCR4 showed that the majority of the cells were double positive after incubation with Th2-polarizing cytokines (Fig. 2 B), but not with Th1 cytokines (data not shown). With this protocol, all cells expressed both receptors simultaneously, although there was a variation in the degree of expression, with definite high and low expressing populations.

The decision concerning the activation state of the Th2 cells to be transferred in vivo is one that deserves comment. Different groups have used different conditions to polarize and activate Th2 cells before transfer in vivo (18–21). In general, these protocols are based on the transfer of Th cells that are polarized for short times in culture. In the system used here, Th cells were maximally polarized and activated to ensure differential chemokine receptor expression. A second reason for the degree of polarization and activation used here was to ensure the induction of multiple pathophysiological endpoints after transfer in vivo. When interpreting the results obtained in this report and comparing them with results obtained in others, it is critical to factor in and to compare such possible differences in activation and polarization of the transferred T cells, which could well represent different stages of disease initiation and progression and/or the cellular basis of different etiologies resulting in the same final chain of pathophysiological events.

To determine the expression pattern of CCR3 and CCR4 and their ligands in lung tissue after adoptive transfer, we performed PCR in pools of mRNA extracted from lungs at days 4 and 7 after Th cell transfer. We found that CCR3 and its ligand, eotaxin, are upregulated in lung RNA isolated after serial OVA challenge of mice after Th2 cell transfer, but not after Th1 transfer (Fig. 2 C). Higher CCR4 mRNA levels were also seen in mice after Th2 cell transfer compared with Th1 transfer. MDC mRNA was expressed after challenge in both Th1 and Th2 recipient mice, presumably since the main cell type producing this chemokine is the macrophage (10). We did not find any significant TARC expression after induction of pulmonary inflammation (data not shown); thus, MDC was used as the ligand for functional studies. The principal site of CCR3 expression is likely to be on eosinophils, and of CCR4 on macrophages, but these receptors have also been found on Th2 cells in vitro (5, 11).

To localize the expression of CCR3 and CCR4 with Th2 cells in vivo, we used immunohistochemical staining to determine that both $CCR3^+$ and $CCR4^+$ Th2 cells were indeed present in the lung after intravenous transfer of antigen-specific Th2 cells and subsequent allergen challenge (Fig. 2 D). Moreover, there was a greater proportion of Th2 cells that expressed CCR3 rather than CCR4 on day 4, whereas the converse was true for day 7, with a greater proportion of Th2 cells expressing CCR4 rather than CCR3 (Fig. 2 D). There were no CCR3- or CCR4-expressing effector Th cells after transfer of Th1 cells (data not shown). When this analysis was performed at day 14 (after 13 in vivo antigen stimulations), the vast majority (>95%) of Th2 cells found in the lung expressed CCR4, whereas there were only a small number (<5%) of CCR3-expressing Th2 cells (Fig. 2 D).

CCR3/Eotaxin and CCR4/MDC Function in a Coordinated Manner to Promote Th2 Cell Recruitment In Vivo. Based on this differential expression of CCR3 and CCR4, we formulated the hypothesis that CCR3/eotaxin and CCR4/MDC pathways play differential, coordinating roles in the development of pathology during allergic reactions. To test this hypothesis, we used neutralizing Abs to block the ligands for CCR3 and CCR4, eotaxin and murine MDC, respectively, after acute or repeated antigen challenge in mice injected with antigen-specific Th cells. It should be kept in mind that, although eotaxin is the main described ligand for CCR3 in both mice and humans, other chemokines such as monocyte chemoattractant protein 3 (MCP-3) have been shown to exert their effects in vitro at least partially through CCR3 binding. Similarly, TARC is yet another known ligand for CCR4. Therefore, blockage of eotaxin and MDC might not necessarily be equivalent to blockage of CCR3 or CCR4.

We have found previously that the antieotaxin and anti-MDC Abs used in this study block the specific migration of CCR3- or CCR4-expressing cells, both in vitro and in vivo (7, 13). Mice were killed on day 4 or 7, and the migration of antigen-specific Th cells to the lung was determined histologically. The donor Th1 and Th2 cells expressed a clonotypic TCR recognized by an mAb, making it possible to

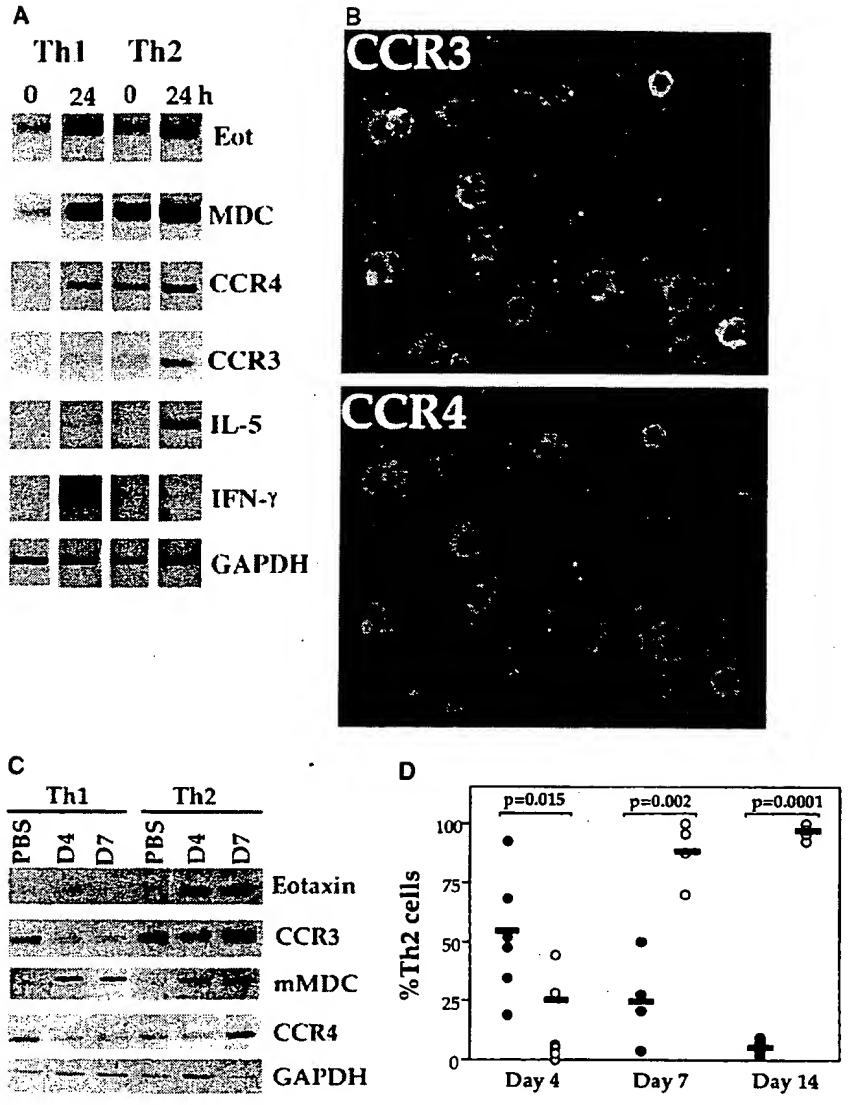

Figure 2. Chemokine receptor expression in donor Th cell populations, and in lungs after Th cell transfer. (A) Expression of chemokine receptors CCR3 and CCR4 and their respective ligands, eotaxin and MDC, were determined by PCR in effector Th cells after three rounds of polarization and activation with anti-CD3 Ab. Polarity of Th cells was checked by expression of IL-5 and IFN-γ in each population. (B) Protein expression of CCR3 and CCR4 was confirmed by immunohistochemical staining of cytospins prepared from third-round polarized cells. (C) Expression of chemokine receptors CCR3 and CCR4 and their respective ligands, eotaxin and MDC, was determined by PCR in lung RNA pooled from three mice transferred with either Th1 or Th2 cells and challenged with PBS or OVA. Levels were compared with those of the housekeeping gene, GAPDH. (D) Relative proportions of $CCR3^+$ and $CCR4^+$ Th2 cells in allergic lung tissue. CCR3- and CCR4-expressing Th2 cells were enumerated by locating an infiltrate in each KJ126-stained section and counting the percentage of KJ126-stained cells that were either $CCR3^+$ or $CCR4^+$ in serial sections. Sections were counted in lungs obtained at day 4 ($n = 6$), 7 ($n = 4$), or 14 ($n = 5$).

distinguish donor (antigen-specific) cells from host Th cells (17). We found that although the total number of CD4 cells was unaffected by blockage of either CCR3 or CCR4 ligands, the percentage of antigen-specific Th2 cells decreased by at least 50% (Fig. 3). In contrast, blockage of eotaxin or MDC had no effect on the migration of antigen-specific Th1 cells (Fig. 3 B). However, the specific effect of blocking one pathway versus the other differed at each time point (as discussed in detail below). These data show that eotaxin and MDC interactions with their specific receptors in vivo are critical for antigen-specific Th2 cell recruitment to the lung in this model.

To determine whether this selective blockage of antigen-specific Th cells by distinct chemokines affected eosinophil infiltration, BHR, and BAL cytokine production, we measured these parameters on day 4 after blockage with antieotaxin, or on day 7 after neutralization of MDC. Eosinophilia was decreased by blockage of either eotaxin or MDC (Fig. 4 A). This may stem from interference in the interaction of eotaxin with CCR3 on eosinophils, but also with CCR3 on Th2 cells, since the initiating event in eosinophil accumulation in this model occurs as a direct result of antigen-specific Th2 cell migration. BHR was also reduced after blockage of either eotaxin or MDC (although the latter did not reach statistical significance; Fig. 4 B). The decreased migration of antigen-specific Th cells also impaired the production of Th2-type cytokines, with significant decreases in IL-4 after blockage of eotaxin or MDC (Fig. 4 C). Neither treatment had any effect on IL-5 production. These results demonstrate unequivocally that Th2 cells alone can initiate pulmonary inflammation and that the recruitment of these essential cells via the CCR3/eotaxin (day 4) and CCR4/MDC (day 7) pathway is a critical event in the development of AAD. In this regard, it is important to note that eosinophilia and BHR were only moderately reduced (~35% inhibition of eosinophilia; data

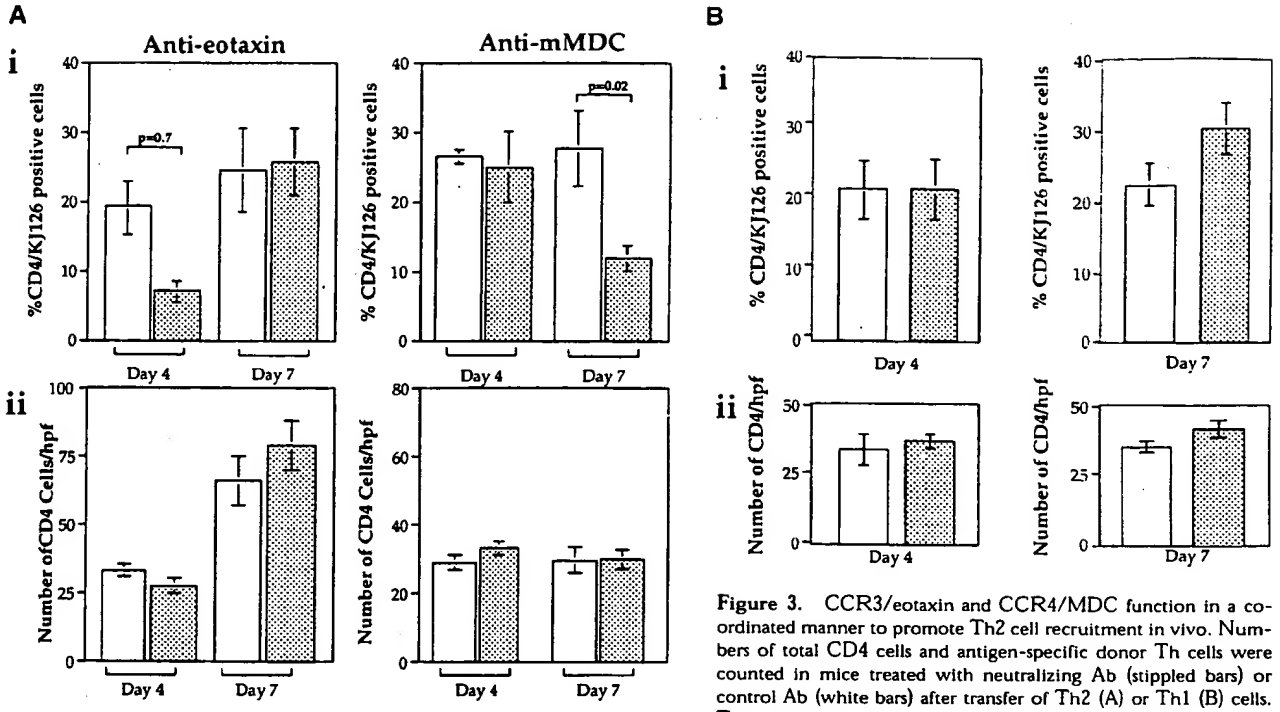

Figure 3. CCR3/eotaxin and CCR4/MDC function in a coordinated manner to promote Th2 cell recruitment in vivo. Numbers of total CD4 cells and antigen-specific donor Th cells were counted in mice treated with neutralizing Ab (stippled bars) or control Ab (white bars) after transfer of Th2 (A) or Th1 (B) cells. The percentage of antigen-specific cells was quantified by counting at least 250 $CD4^+$ cells in each section, and then counting the number of $KJ126^+$ cells in serial sections at a magnification of 400 for 10 mice in each group from two separate experiments (i). Total numbers of $CD4^+$ cells per hpf are shown (ii).

not shown) on day 7 after blockage of eotaxin (compared with ~75% inhibition of eosinophilia at day 4; Fig 4), indicating that other chemokines in addition to eotaxin participate in the final recruitment of eosinophils in vivo (data not shown).

The CCR4/MDC Axis Is Dominant in Mediating Recruitment of Th2 Cells after Repeated Allergen Exposure. We have shown that CCR3/eotaxin and CCR4/MDC mediate selective recruitment of antigen-specific Th cells during the allergic process. However, our results also show that these axes are important at different stages of the disease process. In our model, the CCR3/eotaxin pathway was critical in mediating the recruitment of Th2 cells after initial antigen stimulation in vivo, as determined by effective blockage at day 4. However, after repeated antigen stimulation (on day 7), the CCR3/eotaxin axis was superceded by the CCR4/MDC pathway, which was critical for Th2 migration by day 7. Further stimulation with antigen results in >95% of Th2 cells expressing CCR4 by day 14, but <5% expressing CCR3. This reinforces the view that chemokines and their receptors function in a tightly controlled, coordinated manner, as has been suggested in active immunization models of pulmonary inflammation (22). The interpretation of the results reported here in the context of the pattern of eotaxin and MDC expression in the lung during the development of AAD after active immunization (13) illustrates the complexity of hypothesizing expression and function correlations. For example, we have reported previously that the peak of MDC expression precedes that of eotaxin expression during the course of an active immunization model of AAD (13). Based on the results reported here, we propose that this maximal accumulation of MDC mRNA and protein probably correlates more with an early accumulation of monocytes/macrophages (which produce significant amounts of MDC [13]) in the lung in that particular model than with an early recruitment of Th2 cells. We hypothesize that lower levels of MDC present at later time points (13) are in turn critical for the recruitment of Th2 cells to the airways. Conversely, we hypothesize that maximal levels of eotaxin occurring at later time points during the course of the same active immunization models (7, 13) correlate better with final eosinophil recruitment, whereas lower levels of eotaxin present in the lung at early time points could be key to initiate Th2 accumulation in the lung.

The data presented here also strengthen the hypothesis that Th cells modulate their chemokine receptor expression according to the degree and extent of antigen stimulation and the cytokine milieu. Our experiments indicate that this indeed occurs during the in vivo allergic response. Moreover, our results show that this regulation of expression gives rise to functional consequences. These results are aligned with the data from in vitro experiments that show that Th2 cells lose CCR3 expression and preferentially gain CCR4 expression in response to repeated antigen stimulation (14).

Concluding Remarks. We have taken advantage of a mouse model of AAD based on the adoptive transfer of polarized effector Th cells to determine the functional importance of the chemokine receptor/ligand axes CCR3/eo-

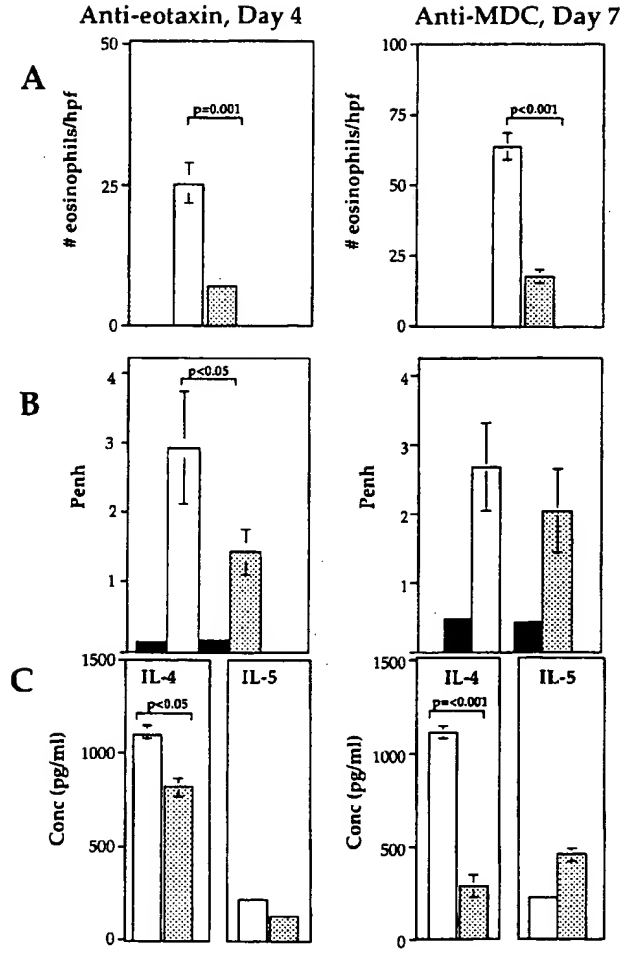

Figure 4. Decreased Th2 cell migration affects development of eosinophilia and BHR. The extent of eosinophilia (A), development of BHR (B), and levels of lavage cytokines (C) were determined in mice transferred with Th2 cells, challenged with OVA, and treated with neutralizing Abs to eotaxin (left) or MDC (right). Mice were given control Ab (white bars) and compared with Ab-treated mice (stippled bars). Numbers of eosinophils were counted in five hpf in cyanide-resistant, peroxidase-stained sections. BHR was measured as described in Materials and Methods and is shown as mean ± P_{enh} for each group of mice after stimulation with methacholine (white and stippled bars) or at baseline (black bars). Levels of IL-4 and IL-5 in BAL were determined by ELISA, and are calculated as mean ± SEM for each group.

taxin and CCR4/MDC in mediating the recruitment of antigen-specific Th2 cells during in vivo allergic reactions. We have shown that both eotaxin/CCR3 and MDC/CCR4 play a critical role in the homing of Th2 cells to the lung after antigen challenge. This finding emphasizes the relevance of previous in vitro results and demonstrates for the first time in vivo that CCR3 and CCR4 not only are markers of Th2 cells, but also have a critical pathophysiological significance in the development of AAD (as determined by their impact in BHR and eosinophilia). Moreover, we have determined that these pathways act in a coordinated cooperative manner, with the CCR3/eotaxin pathway being critical in the acute stages of a response after initial challenge. However, repeated antigen challenge results in an increased frequency of CCR4-expressing Th2 cells. Consequently, the CCR4/MDC pathway ultimately dominates in the recruitment of antigen-specific Th2 cells. Based on these findings, we would like to propose that it is the CCR4/MDC axis which is primarily responsible for the long-term recruitment of antigen-specific Th2 cells to target organs, such as airways, during chronic inflammatory responses in which there is repeated exposure to allergen.

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Intervention of Thymus and Activation-Regulated Chemokine Attenuates the Development of Allergic Airway Inflammation and Hyperresponsiveness in Mice[1]

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Thymus- and activation-regulated chemokine (TARC; CCL17) is a lymphocyte-directed CC chemokine that specifically chemoattracts CC chemokine receptor 4-positive ($CCR4^+$) Th2 cells. To establish the pathophysiological roles of TARC in vivo, we investigated here whether an mAb against TARC could inhibit the induction of asthmatic reaction in mice elicited by OVA. TARC was constitutively expressed in the lung and was up-regulated in allergic inflammation. The specific Ab against TARC attenuated OVA-induced airway eosinophilia and diminished the degree of airway hyperresponsiveness with a concomitant decrease in Th2 cytokine levels. Our results for the first time indicate that TARC is a pivotal chemokine for the development of Th2-dominated experimental allergen-induced asthma with eosinophilia and AHR. This study also represents the first success in controlling Th2 cytokine production in vivo by targeting a chemokine. *The Journal of Immunology,* **2001, 166: 2055–2062.**

CD4-positive T lymphocytes have been suggested to play an integral role in the pathophysiology of bronchial asthma (1, 2). Immunopathologic studies demonstrate an accumulation of CD4-positive T lymphocytes, especially Th2, in airway mucosa (3). This subpopulation of Th cells is capable of producing cytokines such as IL-3, IL-4, IL-5, and IL-13 (4–7), which induce IgE production and eosinophil activation. Thus, CD4-positive Th2 cells orchestrate to induce airway hyperresponsiveness (AHR)[3] as well as the local inflammatory responses (8–10). Numerous inflammatory cells, including eosinophils and lymphocytes, are believed to be recruited to the local sites via a chemotactic gradient. In the processes of transendothelial migration of eosinophils, chemokines such as eotaxin and RANTES act as potent chemoattractants. However, it remains largely unknown how T cells are recruited into the sites of allergic inflammation (11–13) and how T cells control consequent events to induce eosinophil infiltration and bronchial hyperreactivity.

Chemokines, a family of low m.w. proteins that induce specific types of leukocyte chemotaxis, play essential roles in regulating the extravasation and tissue accumulation of a certain cell type during immune and inflammatory responses (14–16). Recent investigations have revealed the existence of a number of novel lymphocyte-directed chemokines (17–19). Among these CC chemokines, thymus- and activation-regulated chemokine (TARC) is the first CC chemokine to be shown to selectively chemoattract T lymphocyte (17). TARC was subsequently identified to be a specific ligand for CC chemokine receptor 4 (CCR4) (18) and to induce chemotaxis of T cells, especially of the Th2 type $CD4^+$ human T lymphocytes (20–22). However, the in vivo pathophysiological roles of TARC remain largely unknown.

In the present studies we addressed the question of whether TARC has any effect on the development of airway eosinophilia as well as AHR in the allergic airway inflammation mimicking those seen in bronchial asthma.

Materials and Methods

Animals

Specific pathogen-free male C57BL/6 mice (6–8 wk old) were obtained from CLEA Japan (Tokyo, Japan) and bred in a pathogen-free mouse facility of the Department of Molecular Preventive Medicine. All animal experiments complied with the standards set out in the guidelines of University of Tokyo.

Reagents

OVA was purchased from Sigma (St. Louis, MO). Hamster anti-mouse TARC mAb 5H5 was prepared as described previously (23, 24). The specificity of this Ab was evaluated by 1) binding assay using ELISA, 2) calcium mobilization assay, and 3) chemotaxis assay as described below.

Direct ELISA

The specificity of monoclonal anti-mouse TARC 5H5 was examined by a direct ELISAs. Recombinant mouse chemokines used for the assay were TARC, liver and activation-regulated chemokine/macrophage inflammatory protein-3α (MIP-3α), macrophage-derived chemokine (MDC), secondary lymphoid chemokine/6Ckine, EBI1-ligand chemokine/MIP-3β, stromal-derived factor-1, RANTES, lymphotactin, MIP-1α, monocyte chemotactic protein-1 (JE), and IL-11 receptor α locus chemokine/cutaneous T cell-attracting chemokine. They were purchased from PeproTech (Rocky Hill, NJ). In brief, ELISA plates (Costar, Cambridge, MA) were coated with recombinant mouse chemokines at a concentration of 2 μg/ml and

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[3] Abbreviations used in this paper: AHR, airway hyperresponsiveness; TARC, thymus- and activation-regulated chemokine; mTARC, mouse TARC; CCR, CC chemokine receptor; MIP-1, macrophage inflammatory protein 1; MDC, macrophage-derived chemokine; BAL, bronchoalveolar lavage; sRaw, specific airway resistance; PBS-T, PBS containing 0.05% Tween 20.

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incubated at 37°C overnight. After washing with PBS containing 0.05% Tween 20 (PBS-T), plates were blocked with PBS containing 1% BSA, 5% sucrose, and 0.05% NaN_3. After washing with PBS-T, 5H5 was added at a concentration of 2 μg/ml and incubated at 37°C for 1 h. After washing with PBS-T, plates were incubated at 37°C for 30 min with biotinylated goat anti-hamster IgG (Vector, Burlingame, CA) at a concentration of 5 μg/ml. After washing with PBS-T, plates were incubated at 37°C for 30 min with HRP-streptavidin (Vector). After washing with PBS-T, bound HRP was developed by tetramethylbenzidine substrate, and OD at 450 nm was measured using a microplate reader.

Calcium mobilization assay

This was conducted using mouse L1.2 pre-B cells stably transfected with mouse CCR4 cDNA as previously described (18). In brief, cells were suspended at 1×10^6 cells/ml in HBSS containing 1 mg/ml of BSA and 10 mM HEPES, pH 7.4 (HBSS-BSA), and incubated with 3 mM fura-2/AM fluorescence dye (Molecular Probes, Eugene, OR) at room temperature for 1 h in the dark. After washing twice, cells were resuspended at 5×10^6 cells/ml. Cells in 0.1 ml were placed into a fluorescence spectrophotometer (F2000; Hitachi, Tokyo, Japan). Mouse TARC (mTARC; 10 nM) or mouse MDC (mMDC) was added to cells in 0.1 ml in the absence or the presence of 5H5 at the indicated concentrations, and emission fluorescence at 510 nm was measured upon excitation at 340 and 380 nm with a time resolution of 5 points to obtain the fluorescence intensity ratio (R340/380).

Chemotaxis assay

L1.2 pre-B cells stably transfected with mouse CCR4 were washed twice with phenol red-free RPMI 1640 medium containing 1 mg/ml BSA, and 0.1 ml of cell suspension containing 2.5×10^5 cells was applied to each of the upper wells of a Transwell plate (3-mm pore size; Costar). Mouse TARC or mouse MDC at 10 nM was preincubated with or without the indicated concentrations of 5H5 for 30 min and added to the lower wells in a volume of 0.6 ml. After 4 h at 37°C migrated cells were determined by measuring dsDNA with PicoGreen dsDNA quantitation reagent (Molecular Probes). Values were expressed as the percentage of input cells that migrated to the lower wells. All assays were performed in triplicate.

Induction of murine asthma model

Pulmonary eosinophilia in response to OVA was generated in mice as described previously (25, 26). In this experience we modified this model. In brief, the murine model of lung eosinophilia used here consists of an initial phase of sensitization and a second phase of induction of the allergic response. Mice were first sensitized with i.p. injection of OVA (0.1 mg/mouse) in 0.2 M PBS/alum (Sigma) on days 1 and 8. The mice were challenged by inhalation of aerosolized 1% OVA for 20 min on days 15–21 to induce the response. At different times after the last allergen challenge, animals were killed under anesthesia with barbiturate. PBS (i.p. and aerosolized) was administered to mice on a similar schedule as in negative controls.

Effect of anti-TARC Ab

In the series of blocking experiments, mice were injected with neutralizing mAb against mTARC 5H5 (50 μg/mouse i.p.) 30 min before OVA administration on days 8–21, and then analyzed 6 h after allergen challenge on day 21. OVA-treated control mice were injected with the same amount of control Ab (hamster Ig fraction; Dako, Santa Barbara, CA) at the same time points as during the treatments. The dose and time schedules of Ab treatment were basically decided according to previous reports with similar experimental strategy (27).

Bronchoalveolar lavage (BAL)

BAL was performed as previously described (11). Briefly, at various time points after the last aerosol exposure, the lungs were lavaged via a tracheal cannula with 0.7 ml of PBS three times. The recovered BAL fluid was immediately centrifuged (1000 rpm, 2 min, 4°C), and cells in BAL fluid were washed twice and resuspended in 1 ml of PBS. The number of cells was determined by hemocytometer. Samples were applied to glass slides by cytocentrifugation (5×10^5 cells/slide), air-dried for 10 min, and then subjected to Wright-Giemsa stain (Fisher Diagnostics, Pittsburgh, PA). The percentages of eosinophils, lymphocytes, neutrophils, and macrophages were determined by counting at least 500 cells/slide using standard morphologic criteria.

Histology

Lung specimens were fixed in 10% neutrally buffered formalin and paraffin embedded. Deparaffinized sections (3 μm thick) were stained with hematoxylin and eosin and analyzed under a light microscope.

Immunohistochemistry

Lung specimens were embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN), frozen in liquid nitrogen, and cut by a cryostat into 7-μm-thick sections. After inhibition of endogenous peroxidase activity (28), the sections were incubated with hamster anti-mouse TARC mAb (5H5) or rat anti-mouse CD4 mAb (RM4-5; PharMingen, San Diego, CA). Hamster anti-mouse TARC mAb (5H5)-treated sections were incubated sequentially with HRP-conjugated anti-hamster IgG (Southern Biotechnology Associates, Birmingham, AL). Rat anti-mouse CD4 mAb (RM4-5; PharMingen)-treated sections were incubated with alkaline phosphatase-labeled anti-rat IgG and anti-hamster IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), respectively. After visualization with 3,3′-diaminobenzidine (Wako Chemicals, Dallas, TX) or alkaline phosphatase substrate kit I (Vector), slides were counterstained with Mayer's hematoxylin. Control hamster (Rockland, Gibertsville, PA) and rat (Sigma) IgG did not stain the same samples in any experiments (10).

Fluorescent immunohistochemistry

To better identify the cell types that were stained for TARC protein, we performed studies with fluorescent microfluorographs. After inhibition of endogenous peroxidase activity, the sections were incubated with hamster anti-mouse CD11c mAb (N418, Serotec, Oxford, U.K.). Hamster anti-mouse CD11c mAb (N418, Serotec)-treated sections were incubated with alkaline phosphatase-labeled anti-hamster IgG (Jackson ImmunoResearch Laboratories). After visualization with alkaline phosphatase substrate kit I (Vector), the sections were washed with water and PBS. After inhibition of endogenous peroxidase activity, the sections were incubated with hamster anti-mouse TARC mAb (5H5). Hamster anti-mouse TARC mAb (5H5)-treated sections were incubated sequentially with FITC-conjugated anti-hamster IgG (Southern Biotechnology Associates). To enhance the fluorescent staining, the sections were incubated with FITC-conjugated anti-FITC IgG (Southern Biotechnology Associates). CD11c mAb (red) and TARC mAb (green) fluorescence could be examined simultaneously under epifluorescence microscope at a wavelength exciting FITC (490 nm). Control hamster IgG (Rockland) did not stain the same samples in any experiment. In these studies TARC expression was stained in green, whereas CD11c-positive cells were stained in red. In addition, negative cells were visualized by a transillumination with a green filter (29).

Chemokine and cytokine gene expression analysis in the lung

Total RNA was isolated from lung specimens using RNAzol (Biotecx, Houston, TX), according to the manufacturer's instructions, reverse transcribed into cDNA, and amplified. The levels of cytokine and chemokine expressions were determined with the novel method of real-time quantitative PCR using the ABI 7700 sequence detector system (PE Applied Biosystems, Foster City, CA) (18). The sense primer for TARC was 5′-CAGGAAGTTGGTGAGCTGG TATA-3′, and the antisense primer was 5′-TTGTGTTCGCCTGTAGTG CATA-3′. The sense primer for GAPDH was 5′-AGTATGACTCCACTC ACGGCAA-3′, and the antisense primer was 5′-TCTCGCTCCTGGAAGA TGGT-3′. The sense primer for eotaxin was 5′-AGAGCTCCACAGCGC TTCTATT-3′, and the antisense primer was 5′-GGTGCATCTGTTGTTGGT GATT-3′. The sense primer for RANTES was 5′-CATATGGCTCGGACA CCACT-3′, and the antisense primer was 5′-ACACACTTGGCGGTTCCT TC-3′. The sense primer for MDC was 5′-TCTGATGCAGGTCCCTATGGT-3′, and the antisense primer was 5′-TTATGGAGTAGCTTCTTCACCCAG-3′. The reaction master mix containing a cDNA sample was prepared according to the manufacturer's protocols to yield final concentrations of 1× PCR buffer A; 200 mM dATP, dCTP, and dGTP; 400 mM dUTP; 4 mM $MgCl_2$; 1.25 U AmpliTaq DNA polymerase; 0.5 U Amp-Erase uracil-*N*-glycosylase; and 200 mM of each primer. The reactions also contained the following target hybridization probes (100 mM each). TARC probe was 5′-ATGCCATCGTGTTTCTGACTGTC CAGG-3′. GAPDH probe was 5′-AACGGCACAGTCAAGGCCGAGAAT-3′. Eotaxin probe was 5′-TGCTCACGGTCACTTCCTTCACCT-3′. RANTES probe was 5′-CAAGTGCTCCAATCTTGCAGTCGTG-3′. MDC probe was 5′-CCAATGTGGAAGACAGTATCTGCTGCCA-3′. The probe was labeled with a reporter fluorescent dye, FAM (6-carboxyfluorescein), at the 5′ end. The thermal cycling conditions included 50°C for 2 min and 95°C for 10 min, followed by 50 cycles of amplification at 95°C for 15 s and 55°C for 1.5 min for denaturing and annealing-extension, respectively. The PCR products were also examined by 2% agarose gel electrophoresis. After ethidium bromide staining, bands were visible only at the expected m.w. for each target mRNA product.

Measurement of cytokine production

The release of cytokines in anti-TARC Ab-treated sensitized mice or control Ab-treated sensitized littermates was determined by ELISA. BAL fluids were collected 48 h after Ag challenge on day 21. BAL fluids concentrated by freeze-drying were assayed using commercially available ELISA kits for IL-4, IL-13, and IFN-γ (Endogen, Boston, MA). Absorbance values were converted to the concentration of each cytokine in the BAL fluid (picograms per milliliter) by interpolation to the respective standard curve. The detection limits of the assay for IFN-γ, IL-4, and IL-13 were 8, 9, and 9 pg/ml, respectively.

Measurement of specific airway responsiveness (sRaw)

We measured the sRaw (centimeters of H_2O per liter per second) in unanesthetized mice with the double-chamber plethysmograph (27) on day 23. The noninvasive technique is based on measurement of the time delay between thoracic and mouth volume changes, and we calculated the airway resistance (30, 31). Airway responsiveness to i.v. methacholine challenge was defined by the sRaw. In brief, mice were positioned in the double chamber. Preliminary experiments demonstrated a significant dose-response relationship between the methacholine dose and the sRaw, where 50 mg/kg of methacholine seemed an optimal dose (data not shown). After establishment of a stable state, methacholine was injected i.v. (50 mg/kg), and sRaw was measured for 5 min.

Measurement of the number of CD4-positive cells

CD4-positive cells were quantified in the area 100 μm beneath the epithelial basement membrane in several nonoverlapping high power fields until all the available area was covered. The final result, expressed as the number of positive cells per square millimeter, was calculated as the average of all cellular counts.

Statistical analysis

Results are expressed as the mean ± SE. Statistical significance analyses were performed unless otherwise indicated by two-way ANOVA, and multiple comparisons were made by Fisher's test. $p < 0.05$ was accepted as statistically significant.

Results

Specificity of anti-mouse TARC mAb

The specificity of anti-mouse TARC mAb 5H5 was first examined by a direct ELISA. 5H5 bound only to recombinant mouse TARC protein but not to other tested mouse CC chemokines, including MDC, liver and activation-regulated chemokine/MIP-3α, secondary lymphoid chemokine/6Ckine, EBI1-ligand chemokine/MIP-3β, stromal-derived factor-1, RANTES, lymphotactin, MIP-1α, MCP-1 (JE), and IL-11 receptor α locus chemokine/cutaneous T cell-attracting chemokine. Furthermore, we checked the cross-reactivity of 5H5 with mMDC by measuring the calcium mobilization and chemotaxis in mouse L1.2 pre-B cells that were stably transfected with mouse CCR4 cDNA. 5H5 completely inhibited mouse TARC-induced calcium mobilization (Fig. 1*A*) and chemotaxis (Fig. 1*B*). In contrast, such inhibition was not seen in mouse MDC-induced calcium mobilization or chemotaxis. Thus, 5H5 was concluded to be a highly specific neutralizing mAb to mouse TARC and was used for in vivo administration and the immunohistochemical studies described below.

Increase in lung TARC mRNA levels in a murine model of asthma

To evaluate the changes in TARC mRNA expression during the development of a murine model of asthma, total lung RNA was extracted 3, 6, 24, and 48 h after the last Ag inhalation (on day 21), and the levels of TARC mRNA were examined by a real-time quantitative PCR. Lung TARC mRNA expression was detectable in the untreated lung, which was significantly increased at 3–6 h after the last OVA challenge, and the levels were subsequently enhanced up to 24 h (Fig. 2).

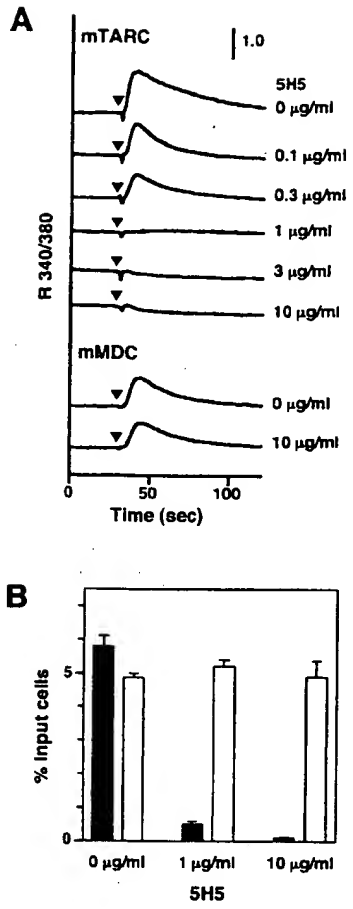

FIGURE 1. Monoclonal anti-mTARC 5H5 had neutralizing effects on mouse TARC, but not mouse MDC. *A*, Mouse TARC and MDC induced calcium mobilization in mouse L1.2 pre-B cells stably transfected with mouse CCR4 cDNA. Mouse TARC or MDC (10 nM) was added to cells in the absence or the presence of 5H5 at the indicated concentration, and emission fluorescence at 510 nm was measured with a time resolution of 5 points. 5H5 blocked mTARC-induced calcium mobilization at <1 μg/ml, but 5H5 could not inhibit mMDC-induced calcium mobilization at 10 μg/ml. *B*, Cell migration was measured using mouse L1.2 pre-B cells stably transfected with mouse CCR4 cDNA. Mouse TARC (■) or mMDC (□) at 10 nM was preincubated with or without the indicated concentration of 5H5 for 30 min. 5H5 could inhibit TARC-induced chemotaxis at 1 and 10 μg/ml, but could not inhibit MDC-induced chemotaxis at 10 μg/ml. Values were expressed as the percentage of input cells that migrated to the lower wells. The mean ± SE of three independent experiments is shown.

Detection of TARC protein by immunohistochemistry

To confirm the production of TARC protein and to identify the producing cells in the lung, immunohistochemical staining was performed. Bronchial epithelial cells specifically expressed TARC in the lung of untreated animals (Fig. 3*B*). We studied TARC expression by immunohistochemical analysis 3, 6, 24, and 48 h after the last Ag inhalation. The results showed that the expression peaked at 6 h (data not shown). In the OVA-treated group, there was strong staining for TARC, mainly in bronchial epithelium, peribronchial lesions, and infiltrating cells (Fig. 3*C*). To better identify the cell types that were stained for TARC protein expression, we performed studies using fluorescent microfluorographs. We chose CD11c, since TARC is known to be preferentially produced by DC, and CD11c (leukocyte integrin CR4 α subunit) has been used as a marker for most dendritic cells (32). TARC expression was stained in green, and CD11c-positive cells were

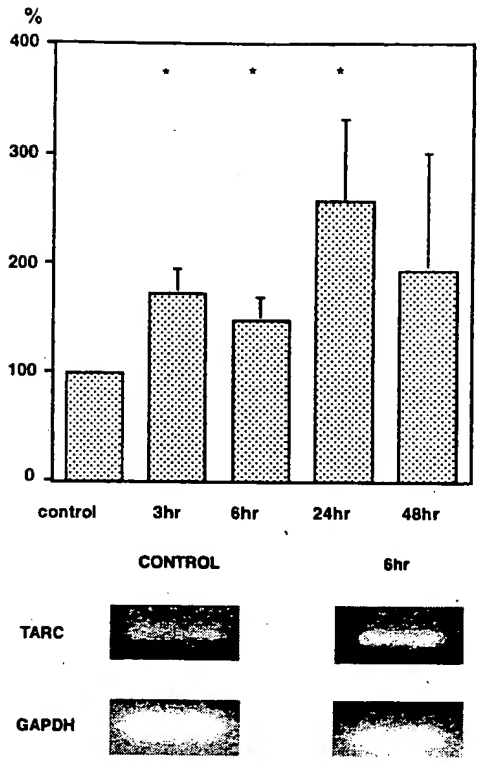

FIGURE 2. Increase in TARC expression in murine models of asthma. *Upper panel*, Real-time quantitative PCR analysis of TARC mRNA expression in the lung. We isolated total RNA from the lung at the following points: untreated and 3, 6, 24, and 48 h after OVA exposure. Total RNA was reverse transcribed with reverse transcriptase and amplified by a real-time quantitative PCR for TARC and GAPDH according to the manufacturer's instructions. The amount of TARC was normalized to the level of GAPDH at each time point. The quantity of TARC mRNA was expressed relative to the calibrator. This result represents four independent experiments. *Lower panel*, Representative results of RT-PCR visualized on 2% gel electrophoresis. *, Values significantly different from those of the PBS groups ($p < 0.05$, by one-way ANOVA followed by Fisher's least significant difference test for multiple comparisons).

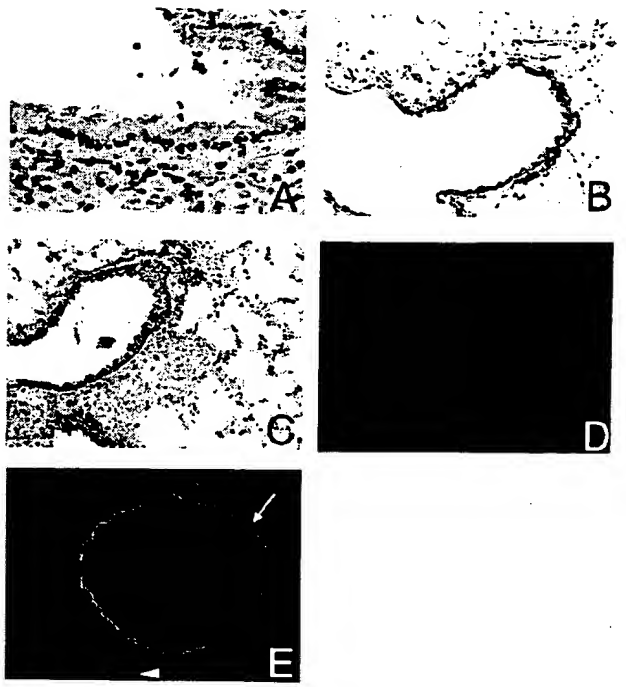

FIGURE 3. Immunohistochemical detection of TARC in untreated (*A* and *B*) and OVA-treated (*C*) mice. Sections of lungs were obtained from untreated mice and OVA-treated mice 6 h after the last Ag inhalation and were fixed and stained with anti-TARC Ab (*B* and *C*) or control anti-hamster IgG Ab (*A*), followed by hematoxylin counterstaining. After immunostaining using anti-TARC mAb, each section was observed at ×100 (*A–C*). *D*, A negative control for OVA-treated mice (1 h after OVA exposure) is shown at ×100. Control hamster IgG did not stain the same sample. *E*, Fluorescent microfluorographs of TARC (green fluorescence) and CD11c (red fluorescence) from OVA-treated mice (1 h after OVA exposure) is shown at ×100. Peripheral bronchial epithelial cells (white arrow) and endothelial cells (white arrowhead) are stained in green.

stained in red. Peripheral bronchial epithelial cells and endothelial cells (Fig. 3*E*) were stained in green, namely those expressing TARC protein. In contrast, CD11c-positive cells adjacent to this kind of structural cell were rarely stained in yellow (double positive).

Anti-TARC Ab attenuated pulmonary eosinophilia in murine asthma models

To evaluate the specific contribution of TARC to the development of lung inflammation in this OVA model, blocking experiments of this chemokine were performed using specific neutralizing Ab. First, we examined the cell profiles of BAL fluids in groups of anti-TARC Ab-treated and control Ab-treated mice. There were marked increases in total cell number, mostly eosinophils, but also macrophages and lymphocytes, in BAL fluids obtained from OVA-treated mice as described previously (11) (Fig. 4). Control Ab did not affect any of the changes induced by OVA treatment in this model of asthma (Fig. 4). Treatment with anti-TARC Ab strikingly decreased the total cell number and the number of eosinophils as well as lymphocytes recovered in the lavage fluid compared with those in the group treated with control Ab (Fig. 4). In contrast, the number of macrophages was not changed by treatment with anti-TARC Abs. These results established that TARC played a pivotal role in the induction of lymphocyte and eosinophil infiltration in the airways.

Histological changes by anti-TARC Ab treatment

In accordance with the changes found in BAL fluid preparations, neutralization of TARC reduced the number of infiltrating cells into the lung in response to OVA, most of which appeared to be mononuclear lymphocytes and eosinophils by hematoxylin-eosin and Wright-Giemsa stainings (Fig. 5, *A–D*).

Anti-TARC Ab attenuated Ag-induced AHR in a murine model of asthma

Eosinophilic inflammation is clearly a hallmark of allergic asthma, and considerable evidence suggests an association between pulmonary eosinophil infiltration and AHR in human asthma (33). To determine the role of TARC in the development of allergen-induced AHR, measurements of airway reactivity to i.v. methacholine were performed on day 23. Animals sensitized and challenged by OVA with the treatment of control Ab showed significantly higher sRaw in response to methacholine compared with saline control animals given control Ab (Fig. 6). The baseline sRaw tended to be higher than that in unsensitized animals, but the difference was not significant. OVA-sensitized and challenged mice treated with anti-TARC Ab showed significantly lower sRaw in response to methacholine compared with those treated with control Ab (Fig. 6). With the anti-TARC Ab treatment, the increase in sRaw was inhibited by 64% ($p < 0.005$), and the sRaw was not

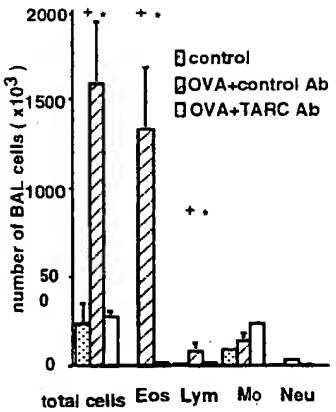

FIGURE 4. The effects of anti-TARC Ab on total cells number and the cell differentials of BAL cells recovered from mice 2 days after the last Ag challenge with either anti-TARC or isotype control Ab treatment. The data are expressed as the mean ± SE. Eos, eosinophils; Lym, lymphocytes; Mϕ, macrophages; Neu, neutrophils. Values shown are the mean ± SE of four to five animals per group. *, Values significantly different from PBS-treated groups ($p < 0.05$). +, Values significantly different from anti-TARC Ab-treated groups ($p < 0.05$).

significantly different from that in the control group (saline injection and saline inhalation) at each time point after methacholine injection. The difference in baseline airway resistance is due to Ag exposure without methacholine. These results indicate that the development of Ag-induced AHR was significantly decreased with anti-TARC Ab.

Anti-TARC Ab suppressed the accumulation CD4$^+$ cells in the lung

To elucidate whether anti-TARC mAb inhibited T lymphocyte infiltration in the lung, we also evaluated the number of CD4$^+$ T cells in the airways by anti-TARC Ab treatment. Anti-TARC mAb treatment markedly decreased the degree of infiltration of CD4$^+$ T cells (number of CD4-positive cells per square millimeter, 58.5 ± 6.19 (±SEM) and 26.5 ± 3.00 (±SEM) in control Ab group and, anti-TARC Ab group, respectively; $p < 0.001$, by Student's *t*es t; Fig. 5, *E* and *F*).

Anti-TARC Ab selectively suppressed the local concentrations of Th2-type cytokines in the lung

Th2 cytokines such as IL-4 and IL-13 are required for pulmonary eosinophilia and induction of AHR (9, 33). To determine whether the blockage of TARC shows any effect on the local production of these cytokines in this model of asthma, we measured the levels of IL-4 and IL-13 as well as that of a Th1-type cytokine IFN-γ. BAL fluids were obtained 48 h after last OVA inhalation, and IL-4 and IL-13 levels were significantly increased in the OVA treatment group (Fig. 7). Blockage of TARC significantly decreased OVA-induced production of these two cytokines. The levels of IFN-γ in BAL fluids were not statistically different between the groups given control Ab treatment and those given TARC Ab treatment at 48 h after the last OVA inhalation (Fig. 7).

Effect of anti-TARC Ab on chemokine expression in the lung

To evaluate the changes in chemokine expression during the development of a murine model of asthma, the levels of eotaxin, RANTES, and MDC mRNA were examined by a real-time quantitative PCR. Lung eotaxin mRNA expression was detectable in the untreated lung, which was significantly increased at 6–24 h after the last OVA challenge, and the levels were suppressed by

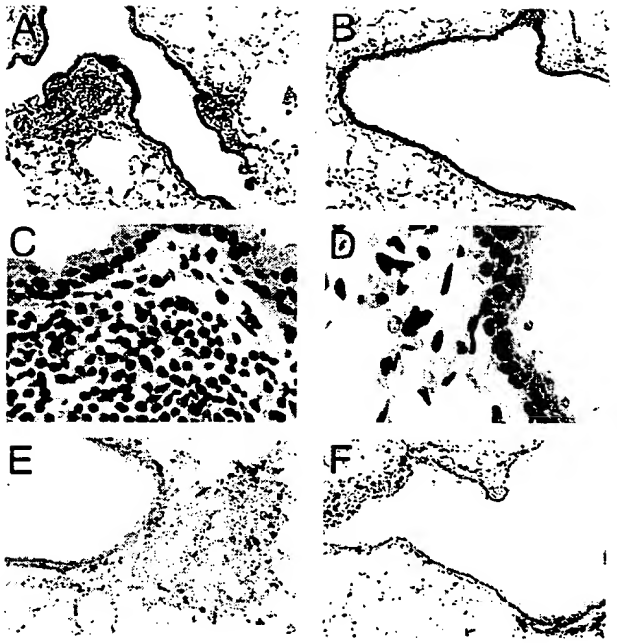

FIGURE 5. OVA-induced histological changes and the effect of chemokine blockage. On day 23 lung tissue was excised from OVA-treated mice injected with control Abs (*A* and *C*) or anti-TARC Abs (*B* and *D*). Chemokine blockage was performed daily before OVA provocation on days 8–21. After staining, each section was observed at ×25 (*A* and *B*) and ×100 (*C* and *D*). Immunohistochemical analysis for CD4$^+$ T cells in a murine model of asthma. Treatment of OVA-challenged mice with anti-TARC Ab (*E*) showed a marked decrease in the number of infiltrating CD4$^+$ cells compared with those treated with control Ab (*F*). After immunostaining using anti-CD4 mAb, each section was observed at ×100.

anti-TARC Ab (Fig. 8*A*). Lung RANTES mRNA expression was increased, but not significantly, after OVA challenge, and the levels tended to be reduced, but were not significantly changed, by anti-TARC Ab (Fig. 8*B*). MDC mRNA expression was significantly increased after OVA challenge, but anti-TARC Ab treatment did not significantly affect MDC mRNA expression (Fig. 8*C*).

Discussion

In the present study we have demonstrated that 1) the expression of TARC was constitutively seen in the lung and was up-regulated in a murine model of allergic asthma; 2) the specific Ab against TARC attenuated OVA-induced airway eosinophilia; 3) the Ab diminished the degree of AHR; 4) the Ab reduced infiltration of CD4$^+$ cells in the airways; and 5) this Ab also decreased Th2 cytokine levels and eosinophil-chemotactic chemokine expression in the lung. These findings suggested that TARC is a pivotal chemokine for the development of allergen-induced tissue eosinophilia and AHR, which are the most important features of bronchial asthma. To the best of our knowledge, this is the first report clearly indicating the role of TARC in the development of asthma.

A number of clinical studies showed that there was an intense infiltration of inflammatory cells, including T cells, especially CD4$^+$ cells, as well as eosinophils. There was a significant correlation between the number of CD4$^+$ cells in BAL fluids and the degree of AHR in asthmatic patients (34). Increasing evidence suggests that T lymphocytes, in particular CD4$^+$ T cells of the Th2 type, play an essential role in the development of the eosinophilic inflammatory response commonly found in asthma (35, 36). Elevated IL-4, IL-5, and IL-13 levels in bronchial biopsies (36, 37), BAL cells, and blood (37) of allergic asthmatic patients have been

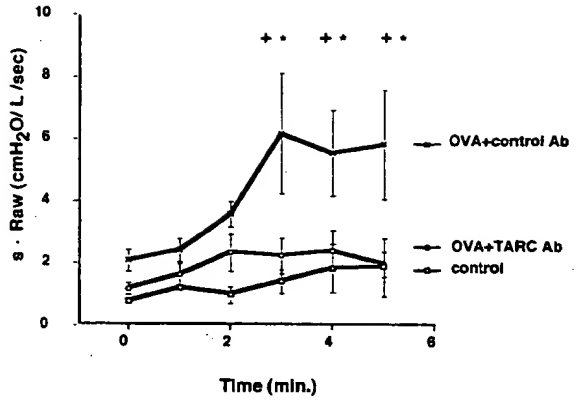

FIGURE 6. Anti-TARC Ab administration diminished the OVA-induced increase in airway reactivity in response to an i.v. methacholine challenge. Control animals (PBS-treated) showed minimal responses to methacholine. OVA-treated mice with anti-hamster IgG (control Ab) showed a significant increase in the airway reactivity to methacholine (sRaw), which was markedly diminished by anti-TARC Ab treatment. Values shown are the mean ± SE of four to five animals per group. *, Values significantly different from PBS-treated groups ($p < 0.05$). +, Values significantly different from anti-TARC Ab-treated groups ($p < 0.05$).

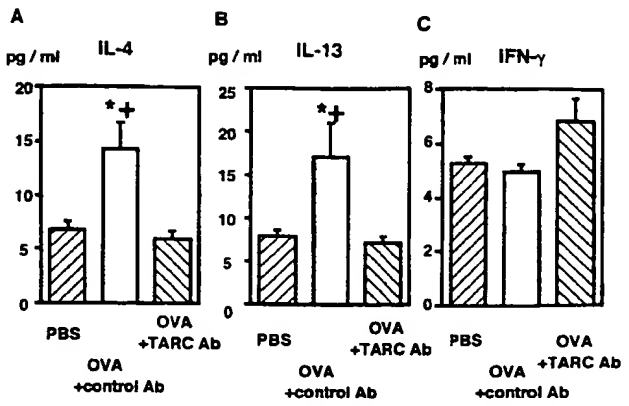

FIGURE 7. Effect of anti-TARC Ab treatment on IL-4 (*A*), IL-13 (*B*), and IFN-γ (*C*) protein levels in BAL fluid of mice 6 h after Ag or PBS challenge. Mice were treated with anti-TARC Ab as described in *Materials and Methods*. Protein levels were analyzed by ELISA. OD readings were converted to picograms per milliliter by comparison with standard curves. Values shown are the mean ± SE ($n = 6$). *, Values significantly different from PBS-treated groups ($p < 0.05$). +, Values significantly different from anti-TARC Ab-treated groups ($p < 0.05$).

reported, and therefore, it is suggested that these Th2-type cytokines play a key role in the eosinophil accumulation and resultant AHR found in asthmatics.

To further elucidate the roles of the Th2 cells and their cytokines, experimental models of asthma have been used. Mice sensitized with OVA showed maximal lung monocyte/macrophage accumulation at early stages of inflammatory response, followed by an increase in eosinophil and T lymphocyte numbers at later stages of the response (11). IL-4, IL-5, and IL-13 (4–7) have been strongly implicated in generating and perpetuating the late phase asthmatic response, including recruitment of activated eosinophils into airways, AHR, and airflow limitation (8–10). Recent reports using T1/ST2-deficient mice (38) strongly suggested that Th2 cells play an essential role in the development of asthmatic airway inflammation. However, it remains unclear how the recruitment of T cells, especially $CD4^+$ cells, into the lung is elicited during allergic inflammation. It is likely that certain chemokines play roles in trafficking effector T lymphocytes into inflamed areas of the lung.

TARC is the first CC chemokine to be shown to selectively chemoattract T lymphocytes (17). TARC was subsequently identified to be a specific ligand for CCR4 (18) and to be a selective chemoattractant for T cells, especially of the Th2 type $CD4^+$ human T lymphocytes (20–22). TARC has been reported to be expressed in dendritic cells and possibly in macrophages. In the present experiment we performed fluorescent microfluorographic studies. TARC was stained in green, and CD11c-positive cells were stained in red as a marker for dendritic cells (32). The results suggested that bronchial epithelial cells and endothelial cells were potential sources of this chemokine (Fig. 3*E*).

Since the number of inflammatory cells in the BAL might be different from that in the tissue itself, we also studied the degree of CD4-positive cell infiltrates in the tissues. Our present findings of blocking experiments with anti-TARC Ab clearly indicated that inhibition of TARC decreased BAL lymphocytes and airway infiltration of CD4-positive T cells, possibly Th2 cells, which produce Th2 cytokines such as IL-4 and IL-13. Decreased local production of these cytokines seemed to attenuate eosinophil accumulation and the following AHR (39, 40) in a number of ways. Since Th2 cells themselves are capable of producing chemokines, a decrease in Th2 cells-derived chemokines such as RANTES might be involved. Besides, recent studies emphasized the importance of airway epithelium-derived chemokines, including eotaxin, in the pathogenesis of asthma (41). Airway epithelial cells produce these chemokines in response to Th2 type cytokines, including IL-4 and IL-13 from T cells (42, 43). Finally, locally recruited eosinophils also produce chemoattractants for themselves, such as RANTES, eotaxin, and lipids such as leukotriene C4 and platelet-activating factor (41).

Treatment with anti-TARC Ab dramatically decreased the number of eosinophils in BAL samples and histology. Studies of mRNA levels of eotaxin in the lung clearly showed a decrease after anti-TARC treatment, whereas the levels of RANTES did not significantly change, suggesting that decreased eotaxin expression might be predominantly involved in this setting.

There is accumulating evidence that shows a critical role for a variety of chemokines in the sequential local migration of inflammatory cells. Gonzalo and colleagues (44) indicated that the coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyper-responsiveness in a murine model of asthma. RANTES expression was up-regulated during the early phase of airway inflammation, suggesting a role in the development of asthma. However, another report (45) failed to show the significance of this CC chemokine in asthma. In our model of asthma, quantitative evaluation of RANTES mRNA expression did not show any significant change (Fig. 8*B*); therefore, its importance remains unknown.

Recent investigations have revealed that CCR3 and CCR4 are expressed on Th2 cells, whereas CCR5 is preferentially expressed on Th1 cells (15, 21, 22, 46, 47). The ligands for CCR4 include a CC chemokine MDC in addition to TARC. MDC not only shares CCR4 with TARC as its specific receptor, but also shows several common features with TARC: it has 32% homology with TARC in amino acid sequence and is a potent chemokine for T cells (48). MDC has been reported to be expressed by dendritic cells, which also produce TARC. Gonzalo et al. (49) described the role of MDC in a murine asthma model similar to ours. In their hands, blocking of MDC by the polyclonal Ab resulted in prevention of AHR associated with significant reduction of infiltratory eosinophils in the lung interstitium, but not in BAL (49). Since the mAb against

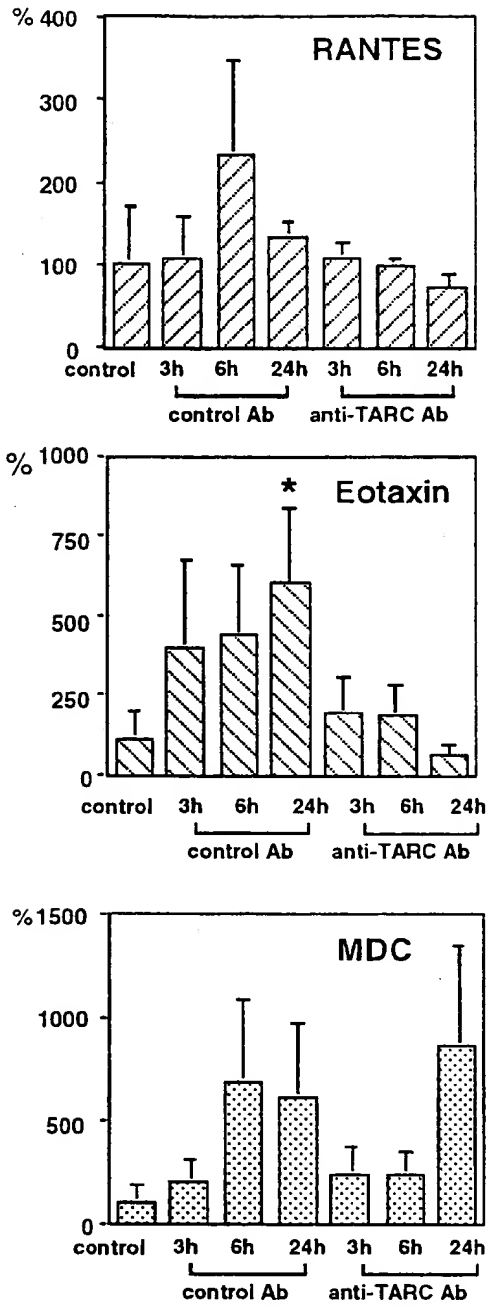

FIGURE 8. Increase in eotaxin expression in murine models of asthma. Real-time quantitative PCR analysis of eotaxin and RANTES mRNA expression in the lung. We isolated total RNA from the lung at the following points: untreated and 3, 6, 24, and 48 h after OVA exposure. Total RNA was reverse transcribed with reverse transcriptase and amplified by a real-time quantitative PCR according to the manufacturer's instructions. The amount of cytokines was normalized to the level of GAPDH at each time point. The quantity of cytokine mRNA was expressed relative to the calibrator. This result represents four independent experiments. *, Values significantly different from those of PBS groups ($p < 0.05$). Significance was determined by one-way ANOVA, followed by Fisher's least significant difference test for multiple comparisons.

TARC used in our experiments was highly specific for TARC and did not cross-react with MDC (Fig. 1, *A* and *B*), our data strongly suggest that TARC is also an essential chemokine for T cells in the development of allergic inflammation in addition to MDC, although further study is necessary to better elucidate the mutual roles of TARC and MDC in T cell migration.

It would be important to study CCR4 expression on $CD4^+$ infiltrating T lymphocytes in the airways. We attempted to study CCR4 expression on BAL cells by FACS, but the commercially available Ab (Santa Cruz Biotechnology, Santa Cruz, CA) detects the C-terminals of intracellular domains of CCR4, and therefore, it was unsuccessful. As for the results obtained by cryostat sections, there was an intense staining for airway epithelial cells and endothelial cells, and accurate evaluation of CCR4-positive T cells were not possible (data not shown).

During the preparation of this manuscript, an important paper appeared related to this study. Chvatchko et al. reported that CCR4 deletion had no effect on a Th2-dependent model of allergic airway inflammation in mice (50). However, we must keep in mind that the findings obtained from their knockout mice cannot always be applied to the actual pathophysiology of the disease, because they are mature animals that have lacked the targeted gene since birth. In our studies we directly assessed the importance of TARC in the development of a murine model of asthma using anti-TARC-specific neutralizing mAb and found that 1) the expression of TARC was constitutively seen in the lung and was up-regulated in murine models of allergic asthma; 2) the specific Ab against TARC attenuated OVA-induced airway eosinophilia, the degree of AHR, and the infiltration of $CD4^+$ cells in the airways; and 3) this Ab also decreased Th2 cytokine levels and eosinophilic cytokine expression in the lung. Recently, it was reported that TARC also binds to CCR8 to induce chemotaxis (51). Therefore, it is possible that TARC might be involved in allergic airway inflammation via binding to CCR8 as well as CCR4.

In conclusion, our results demonstrate that a CC chemokine, TARC, is essentially involved in the development of AHR and eosinophilia through the recruitment of Th2-type CD4-positive T lymphocytes in a murine model of bronchial asthma. Therefore, TARC could be a novel target for intervention therapy of asthma.

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